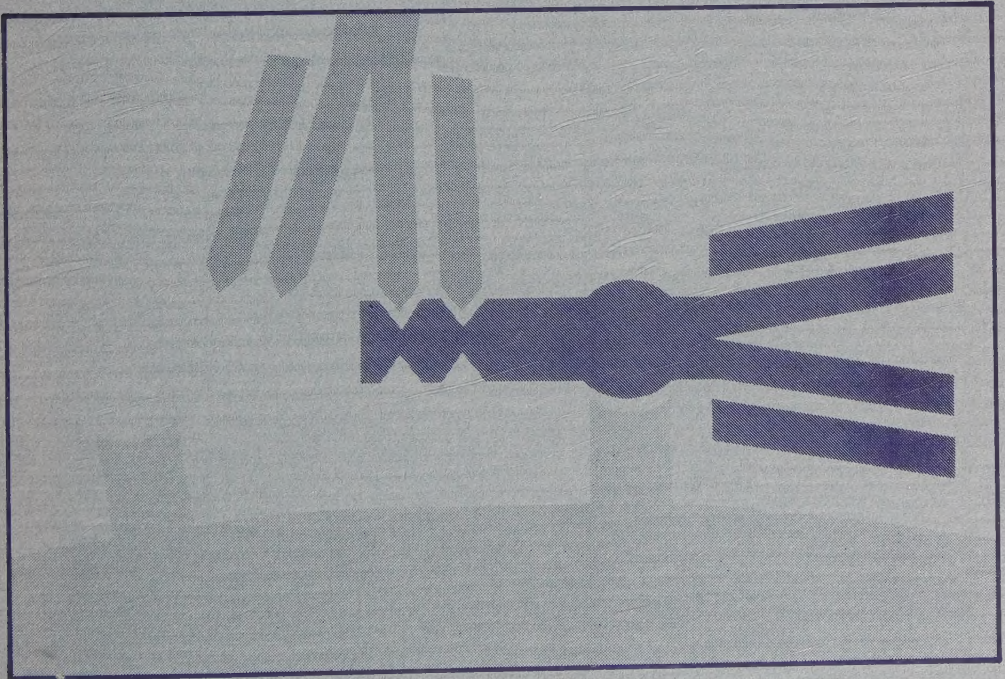


Claës Schalén

IMMUNOGLOBULIN RECEPTORS OF GROUP A STREPTOCOCCI

**Their specificity and
importance for virulence**



Lund 1982

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**Department of Medical Microbiology,
University of Lund,
Lund, Sweden**

1982

This thesis is based on the following publications which will be referred to in the text by Roman numerals:

- I Schalén, C., Christensen, P. & Grubb, R.: Lancefield extract of group A streptococci type 15 acts like an anti-human IgG with restricted specificity. *Acta path. microbiol. scand. Sect. C*, 86:41-43, 1978.
- II Christensen, P., Burova, L.A., Grubb, A., Grubb, R., Samuelsson, G., Schalén, C. & Svensson, M-L.: Interaction of the Fc part of IgG with Lancefield extracts of hemolytic streptococci. Strain specificity and activity. *Acta path. microbiol. scand. Sect. C*, 87:73-77, 1979.
- III Christensen, P., Grubb, A., Grubb, R., Samuelsson, G., Schalén, C. & Svensson, M-L.: Demonstration of the non-identity between the Fc receptor for human IgG from group A streptococci type 15 and M protein, peptidoglycan and the group specific carbohydrate. *Acta path. microbiol. scand. Sect. C*, 87:257-261, 1979.
- IV Schalén, C.: The group A streptococci receptor for human IgA binds IgA via the Fc-fragment. *Acta path. microbiol. scand. Sect. C*, 88:271-274, 1980.
- V Schalén, C., Christensen, P., Grubb, A., Samuelsson, G. & Svensson, M-L.: Demonstration of separate receptors for human IgA and IgG in group A streptococci type 4. Separation of the solubilized receptors from group- and type-specific antigens, lipoteichoic acid and peptidoglycan. *Acta path. microbiol. scand. Sect. C*, 88:77-82, 1980.
- VI Grubb, A., Grubb, R., Christensen, P. & Schalén, C.: Isolation and some properties of an IgG Fc binding protein from group A streptococci type 15. *Int. Archs Allergy appl. Immun.* In press.
- VII Burova, L.A., Christensen, P., Grubb, R., Jönsson, A., Samuelsson, G., Schalén, C. & Svensson, M-L.: Changes in virulence, M-protein and IgG Fc receptor activity in a type 12 group A streptococcal strain during mouse passages. *Acta path. microbiol. scand. Sect. B*, 88:199-205, 1980.
- VIII Schalén, C., Burova, L.A., Christensen, P., Grubb, R., Samuelsson, G. & Svensson, M-L.: IgG F(ab')₂ of rabbit anti-M sera but not the unfractionated sera are bactericidal for some group A streptococci with IgG Fc-receptor activity. Opsonic effect ascribable to anti-IgG. *Acta path. microbiol. scand. Sect. C*, 89:247-252, 1981.



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Introduction

The high prevalence of streptococcal infections affecting man - and particularly children - is a world-wide phenomenon (see Rotta, 1974). The acute infections, often taking an epidemic form, as well as their complications such as rheumatic fever and glomerulonephritis, have serious socio-economic consequences. The prevalence of rheumatic fever has decreased markedly in the industrialized countries during the last few decades, though this complication is still the main factor underlying acquired heart valvular disease in many developing countries (Padmavati, 1978; WHO Memorandum, 1978). It is still a matter of controversy whether poststreptococcal glomerulonephritis can lead to chronic renal disease (see Schacht et al., 1976; Potter et al., 1978a). Sporadic cases or minor outbreaks of acute poststreptococcal glomerulonephritis are not infrequently observed in highly developed countries, whereas widespread epidemics are seen mostly in the developing countries (see e.g. Holm et al., 1973; Poon-King et al., 1973).

Despite extensive research, we are not yet able to explain the pathogenesis of rheumatic fever or glomerulonephritis. These non-suppurative complications occur days or even weeks after the triggering infection. It is well established that nephritogenic potential is associated with a limited number of group A streptococcal types (Rammelkamp & Weaver, 1953; Dillon et al., 1974). More recent data have indicated that also rheumatogenicity is to some degree restricted to certain types (Stollerman, 1971; Potter et al., 1978b; Bisno, 1980).

Since long time, attention has been focused on the immune response to streptococci, which in some respects differs from that against other microorganisms (see below). The recently discovered capacity of group A, C and G streptococci to interact with immunoglobulins, irrespective of the antigen-combining sites, also distinguishes these streptococci from most other microorganisms. The present thesis deals with such interactions, and their possible consequences.

Streptococcal Group and Type Antigens

Carbohydrate antigens serve as an accurate indicator by which streptococci from various sources can be distributed into serological groups. As long as half a century ago, five distinct groups, named A - E, were identified during capillary precipitin testing with streptococcal extracts and antisera (Lancefield, 1933). The list of streptococcal groups has since been extended to include most of the alphabet (for review see Jones, 1978). In clinical practice, however, the Lancefield grouping procedure has gradually been replaced during the last decade by more convenient methods, co-agglutination in particular, in which streptococci are agglutinated by antibody-coated staphylococci (Christensen et al., 1973).

Within some serological groups of streptococci, it has been possible to identify various serological types. For instance group A streptococci can be subdivided into types by two principally different antigen sets, the M- and the T-antigens. It was Lancefield (1928) who first described the subdivision of streptococci into M-types. The M-antigens are type-specific antigens in group A streptococci, extractable by acid hydrolysis and destroyed by trypsin (Lancefield, 1962); the designations M-protein antigens or M proteins are also used (Fox, 1974). The M-antigens are immunochemically detected by capillary precipitin tests, using absorbed antisera (Swift et al., 1943), or by double radial gel-diffusion, permitting the use of unabsorbed antisera (Rotta et al., 1971). However, M-typing in clinical practice is hampered by the lack of commercially available M-typing sera. Further problems in M-typing arise from cross-reactions among M-types (Wiley & Bruno, 1968), from the alleged occurrence of two M-antigens in certain strains (Wiley & Wilson, 1961) and from other constituents, e.g. the M-associated proteins (Widdowson et al., 1971) and the immunoglobulin receptors.

Alternative assay methods for M-proteins were reviewed by Fox (1974). Strains producing opacity in serum can be M-typed by the specific inhibition of this reaction with type-specific sera, as the opacity factor is type-specific and closely related to M-antigen (Maxted et al., 1974). Another means of M-typing is based on the "long chain reaction", i.e. creation of elongated streptococcal chains by type-specific antisera (Stollerman & Ekstedt, 1957); however, the test is more useful for the determination of type-specific antibodies (Stollerman et al., 1959).

The classic methods adopted for M-typing as outlined are based on a precipitin reaction between solubilized M-protein and type-specific antibodies. Moreover, the indirect bactericidal test is also currently used for typing purposes, taking advantage of the capacity of type-specific antisera to kill the homologous type in the presence of fresh blood; thus, the combined results of precipitation and opsonic tests are used to determine the M-type

(Maxted, 1978; Facklam & Edwards, 1979). It should be emphasized, however, that immunochemically detected cross-reactions between serotypes are not invariably paralleled by the results of opsonic tests (Wiley & Bruno, 1968; Beachey & Cunningham, 1973); recently, the type-specific and anti-opsonic determinants were shown not to be identical (Fischetti et al., 1976; Dale et al., 1980).

T-typing involves slide agglutination of trypsinized cells (Griffith, 1934); here, the T-antigens resist trypsin, whereas the M-antigens are destroyed. Anti-T antibodies afford no protection. Various M-types may have a common T-antigen, e.g. the T12-antigen is found among M-types 12, 22 and 62 (Maxted et al., 1974). Thus, the definite subdivision of group A streptococci depends on the M-antigen. However, T-typing is of great clinical importance, since more than 90 % of unselected strains are T-typable, and since antisera are now commercially available. The number of possible M-types represented by a single T-antigen may be reduced by testing for opacity factor and NADse production, since these enzymes are produced only by certain M-types (Ofek et al., 1971; Maxted & Widdowson, 1972; Ivarsson & Christensen, 1977).

Virulence Factors in Group A Streptococci

The M-protein was early incriminated as a virulence factor in group A streptococci. Todd & Lancefield (1928) found that "the type-specific M is present in the potentially virulent organisms comprising the matt variety of colony" and that "it is not present in the avirulent variant cocci which form glossy colonies". However, it was also found that "matt colonies could occur in two forms, equally rich in type-specific substance one no more virulent for mice than the glossy variant, the other highly virulent". The important and frequently forgotten conclusion was that M-protein was not the sole factor determining virulence: Thus, "virulence is not entirely dependent on the presence or absence of the type-specific substance M; some additional factor is operative". The authors also found that the virulence of a strain for man was not always reflected in the outcome of laboratory tests with mice: many fresh, matt isolates from human sources were avirulent for mice. On the other hand, the virulence for mice could frequently be enhanced by passaging the strain from mouse to mouse, whereas the virulence for man seemed unaltered (Lancefield, 1962). However, no data on the virulence for man of mouse-passaged strains are generally available for obvious reasons.

The resistance of M-antigen-containing strains to phagocytosis by human blood not containing antibodies to the respective M-antigen is demonstrated in the bactericidal test (Maxted, 1956; Lancefield, 1957). The mechanisms underlying the anti-phagocytic action of M-protein are poorly understood. Bisno (1979) has shown that strains devoid of M-protein activate the alternative complement pathway, which may explain why they are readily phagocytosed. This activation seems to be blocked by the presence of M-protein. Molecular mimicry of M-protein with

tropomyosin, present in leukocytes, has been proposed to explain the anti-phagocytic effect of M-protein (Hosein et al., 1979).

Opsonization of strains multiplying in blood is mediated by antibodies which are type-specific, although allegedly not identical with those antibodies which precipitate M-protein (Fischetti, 1977). Type-specific antibodies are known to protect man from streptococcal infection (Lancefield, 1959). Recently, peptic fragments of purified M-protein were successfully used to induce type-specific, protective antibodies in man (Beachey et al., 1979).

These data confirm the classically held view of M-protein as being a virulence factor. However, the intricate problem of streptococcal virulence may have been complicated by the use of resistance to phagocytosis as a means to define strains carrying M-protein (Lancefield, 1958; Wiley & Bruno, 1972). Thus, also the hyaluronic acid capsule was early implicated in resistance to phagocytosis and virulence (Kass & Seastone, 1944; Rothbard, 1948; Hirsch & Church, 1960). Foley & Wood (1959) demonstrated that the capsule was an important factor in resistance to phagocytosis of highly virulent strains. Becker et al. (1973) selected streptococcal strains - by rotation in human blood and by passaging in mice - which were equally rich in M-protein and hyaluronic acid; however only the mouse-passaged variants were found virulent for mice. Becker et al. in agreement with Lancefield (1962) and Wiley & Bruno (1972) concluded that "other as yet unidentified factors, in the presence of M-protein" were necessary for mouse virulence. M-negative but highly mouse-virulent variants, obtained by serial mouse-passaging, were recently reported by Burova (1979); some of the variants thus isolated were further investigated in the present work.

Immune Response to Streptococci with Special Reference to Anti-Immunoglobulins

Two aspects of the antibody response to experimental streptococcal immunization have received particular attention: the induction of monoclonal antibodies and of anti-immunoglobulins. Osterland et al. (1966) found that when rabbits were hyperimmunized with pepsin-treated group A, A-variant and group C streptococcal vaccines, unexpectedly high levels of precipitins against the cell wall carbohydrates appeared. Immunization with group A-variant streptococci, in particular, will give rise to antibodies directed against the peptidoglycan (Karakawa et al., 1967). These antibodies are electrophoretically homogeneous and replace the bulk of serum gammaglobulin (Braun et al., 1969). Genetic influences on the immune response and light chain and subclass distribution have been extensively studied in these systems (see e.g. Eichmann et al., 1971; Perlmutter et al. 1978 a,b).

High levels of anti-immunoglobulins also often occur in anti-streptococcal sera (Eyquem et al., 1959; Aasted, 1974; Pillot & Grangeot, 1975). Both IgG and IgM class anti-IgG are regularly detected (Bokisch et al., 1972; Bokisch et al., 1973a). By

contrast, antipneumococcal sera, although exhibiting high immunoglobulin concentrations, contain low or non-detectable levels of anti-IgG. A cross-reactivity between the peptide side chain of group C peptidoglycan and rabbit IgG was claimed to explain the occurrence of anti-IgG in sera raised against group C streptococci (Bokisch et al., 1973b), but this finding has not been verified.

In addition to streptococci, other bacteria (Abruzzo & Christian, 1961; Christian, 1963) as well as plasmodia (Klein et al., 1970) may elicit production of anti-Ig in immunized animals, although to a lesser extent than streptococci do (Bokisch et al., 1972). It has been suggested that the anti-Ig is provoked by circulating immune complexes (Henney et al., 1965). Another mechanism for anti-Ig production appears to be related to mitogen stimulation (Möller et al., 1980); experimental support for the capacity of e.g. lipopolysaccharide to induce anti-Ig response was provided recently (Dresser & Popham, 1976; Izui et al., 1979).

In studies on acute streptococcal infection in humans, interest has for decades focused mainly on the production of antibodies against extracellular streptococcal factors (Stollerman, 1972) as well as M-protein (Lancefield, 1959) and OF (Maxted et al., 1973). Elevated levels of antibodies against the group-specific carbohydrate were demonstrated in acute infections (Riesen et al., 1976; Aasted et al., 1979) and in poststreptococcal sequelae (Dudding & Ayoub, 1968; Bergner-Rabinowitz, 1979). Antibodies reactive with streptococcal peptidoglycan seem to occur in at least 30 % of normal human sera (Schachenmayr et al., 1975; Schalén & Christensen, 1979), probably reflecting immunological cross-reactions between the peptidoglycans of various bacterial species (Schleifer & Kandler, 1972). A higher frequency of such antibodies has been found in patients with streptococcal sequelae (Braun & Holm, 1970). It is of particular interest that high levels of anti-peptidoglycan antibodies have been detected in patients with juvenile (Heymer et al., 1976) and adult (Schachenmayr et al., 1975) rheumatoid arthritis. However, it should be remembered that anti-Ig can interfere with the methods used for detection of anti-peptidoglycan antibodies (Schalén & Christensen, 1979) as with other antibody determinations (see Salonen et al., 1980). For example the L-agglutination of streptococcal cells, exhibited by most rheumatoid sera (Kalbak, 1948), is ascribable to anti-IgG and not mainly to antistreptococcal antibodies (Christensen et al., 1975).

Anti-IgG resembling classical rheumatoid factors are frequently seen in some chronic human infections (Williams et al., 1972), including subacute bacterial endocarditis (Williams & Kunkel, 1962). In acute rheumatic fever, the occurrence of anti-IgG has been found not to exceed that in normal individuals (see Müller, 1962). However, in acute poststreptococcal glomerulonephritis, a high incidence of anti-IgG was recently reported (McIntosh et al., 1979).

Streptococcal and Other Microbial Immunoglobulin Receptors

Bacteria-free animals are known to produce only traces of immunoglobulins, and the production of immunoglobulins is allegedly to a large extent dependent on the presence of microbes (Gustafsson & Laurell, 1958; Gustafsson & Laurell, 1959). Immunoglobulins are produced not only as a specific response to microbial antigens (Coutinho & Möller, 1974). Various microbes are mitogenic for human B-lymphocytes *in vitro*; e.g. whole bacteria (Banck & Forsgren, 1978), including *Mycoplasma pneumoniae* (Biberfeld, 1977), and Epstein-Barr virus (Rosén et al., 1977). Certain purified bacterial components are also mitogenic for human B-lymphocytes, e.g. lipopolysaccharide from *E. coli* (Primi et al., 1977), protein A (Forsgren et al., 1976; Ringdén & Rynnel-Dagöö, 1978) and peptidoglycan (Dziarski & Dziarski, 1979).

The mitogenic and antigenic effects are important aspects of the interactions between the microorganisms and the immune system. In addition, certain microorganisms have developed the capacity to bind immunoglobulins in "non-conventional" ways. This expression, however, seems to refer to early immunological theories rather than to the evolution of the immune system. Thus, the "conventional" interaction, through the antigen-combining sites, is believed to involve only 1 % of the immunoglobulin molecule (see Grubb, 1970).

Staphylococcal protein A is the bacterial factor first shown to bind IgG via the Fc part (Forsgren & Sjöquist, 1966). Streptococcal receptors for IgG (Kronvall, 1973a; Christensen & Kronvall, 1974; Christensen, 1976) and IgA (Christensen & Oxelius, 1975) have now been extensively studied. IgG Fc-receptors have been identified in *Schistosoma mansoni* (Kemp et al., 1977; Torpier et al., 1979) and, recently, in pathogenic protozoa of the *Trypanosomatidae* (Ferreira De Miranda-Santos & Campos-Neto, 1981). *Herpes simplex* type 1 virions also express IgG Fc-receptors (Para et al., 1980), and such receptors may be induced in cells infected *in vitro* with *Herpes simplex* virus (Watkins, 1964) or cytomegalovirus (Rahman et al., 1976). A binding of IgD by various bacteria was discovered in 1979 by Forsgren & Grubb. Finally, a receptor for bovine IgM Fc in *Brucella abortus* was described recently (Nielsen et al., 1981).

Protein A

Protein A, produced by most strains of *Staphylococcus aureus* (Forsgren, 1970), displays an affinity for the Fc-part of human IgG1, 2 and 4, but not IgG3 (Kronvall & Williams, 1969), as well as for IgG of most mammals but, with rare exceptions, not other vertebrates (Kronvall et al., 1970; Kronvall et al., 1974). Recently, an affinity for human IgG3 Fc of some allotypes, rarely occurring in Caucasians, was described (Ito et al., 1980; Recht et al., 1981). A reaction found between protein A and human IgA was first reported to be limited to the IgA2 subclass (Patrick et al., 1977). Later, affinity was found for both IgA subclasses (Brunda et al., 1979) as well as for IgM (Lind et

al., 1975). However, as a (restricted) affinity for the Fab parts of all Ig classes (except IgD) had been detected these reactions were claimed to be mediated by the Fab regions (Johansson & Inganäs, 1978; Inganäs et al., 1980; Inganäs, 1981).

Protein A also reacts with antigen-bound IgG, and protein A or intact staphylococci can be used to isolate immune complexes (Jonsson & Kronvall, 1974; Hällgren & Wide, 1976; Natali et al., 1980). Hypersensitivity phenomena were elicited in rabbit by injecting protein A combined with human IgG (Gustafson et al., 1967) and, in guinea pig, by protein A alone (Gustafson et al., 1968). The injury to human platelets known to be caused by *Staphylococcus aureus* was recently shown to be mediated by protein A (Hawiger et al., 1979). The first component of the complement system, C1q, interferes with the binding of protein A to IgG (Hällgren & Wide, 1976). Small amounts of protein A activate serum complement similar to aggregated IgG, while large amounts of protein A inhibit the fixation of C1q to IgG (Sjöquist & Stålenheim, 1969; Kronvall & Gewurz, 1970). An inhibitory effect of protein A on the binding of IgG immune complexes to cellular Fc-receptors has been reported (Austin & Daniels, 1976; Rosenblatt et al., 1977). Experiments with mouse lymphoid and macrophage cells showed that complexes of protein A and IgG, as compared with IgG alone, increased the capacity of these effector cells to kill target cells (Sulica et al., 1976; Laky et al., 1978). Soluble complexes of protein A and rabbit IgG anti-sheep red cell antibodies, formed in IgG: protein A ratios of 2:1 or 3:2, were found to behave functionally like IgM antibodies with respect to lysis of sheep red cells in the presence of guinea-pig complement (Langone et al., 1978a). In contrast, complexes formed in an excess of protein A were unable to induce lysis (Langone et al., 1978b). The interaction between protein A and IgG may obviously have various biological consequences.

Streptococcal IgA and IgG receptors

The presence of IgG-receptors in certain group A, C and G streptococci was originally demonstrated by the capacity of the bacteria to agglutinate sheep red cells sensitized by rabbit antibodies and to bind radiolabelled IgG (Kronvall, 1973a; Christensen & Kronvall, 1974). Soluble immune complexes, created *in vitro*, were also able to agglutinate these streptococci, further confirming the existence of IgG receptors (Christensen, 1975). A quantitation of the IgG receptor activity was achieved by estimating the uptake of radiolabelled myeloma protein on intact bacteria under standardized conditions (Christensen & Oxelius, 1974). Among the streptococci, certain strains, such as group A type M1 and group C and G strains, displayed particularly pronounced binding capacity. The IgG receptor was found to bind IgG via the CH2 domain in inhibition experiments with purified human IgG fragments (Christensen et al., 1976a).

Kronvall (1973a) found that one IgG3 myeloma protein out of every two investigated lacked reactivity with a group A

streptococcal strain, whereas both were bound by a group G strain. Otherwise, only minor differences have been detected among the four human IgG subclasses as regards affinity for streptococcal Fc-receptors on intact bacteria (Christensen & Oxelius, 1974; Myhre & Kronvall, 1980b). However, in studies on the binding of IgG subclasses from various mammals, five different types of IgG-binding structure in Gram-positive cocci have been reported: type I, represented by *Staphylococcus aureus*; type II, by group A streptococci; type III, by human group C and G streptococci (Myhre & Kronvall, 1977); type IV, by bovine group G streptococci (Myhre et al., 1979); and type V, by *Streptococcus zooepidemicus* (Myhre & Kronvall, 1980a).

By measuring the binding of radiolabelled IgA myeloma protein to intact group A streptococci, Christensen & Oxelius (1975) demonstrated the presence of an IgA-receptor in strains representing types 4, 11 and 57. In type M4, exhibiting the highest activity, no correlation was found between presence of IgG and IgA receptor in the individual strains, indicating that these represent different structures. Kronvall et al. (1979) detected a reactivity with human IgA in 12 of 14 investigated type 49, group A streptococcal strains, and in individual strains of other serotypes, but not in group C and G streptococci. The group A streptococcal IgA-receptors bind both human IgA subclasses (Myhre & Kronvall, 1979).

While streptococcal Ig-binding substances have not been shown to be released during bacterial growth *in vitro* (Schalén, unpublished observation), various extraction procedures have been described. Christensen & Kronvall (1974) demonstrated the possibility of recovering streptococcal IgG Fc-receptors by heat extraction at 95°C; of the different pH-values tested, pH 10.0 gave the highest yield. Havlíček (1978) found an agglutinating capacity for rabbit IgG-coated sheep red cells in the acid extracts of several types of group A streptococci. Christensen & Holm (1976) found a rich release of IgG Fc-receptors on heating some group A strains at 120°C for 30 min. They were also able to solubilize the IgG Fc-receptors of group A type M1 and M56 and group C streptococci by using group C phage-associated lysin, though the yield was comparatively small. The use of detergents, suitable for the extraction of M-protein (Fischetti et al., 1976), is being studied.

An enhanced binding of human aggregated IgG to some group A streptococci as a consequence of presence of fresh serum, or "early" complement components, was reported by Christensen et al. (1977b). This binding capacity, distinct from IgG Fc-receptor activity, is restricted to some of the types with nephritogenic potential, in particular type 12 streptococci (Christensen et al., 1978; Christensen et al., 1981).

Streptococcal binding of various serum proteins

Several interactions between streptococci and serum proteins other than immunoglobulins have been reported. Thus, human fibrinogen and aggregated human β_2 -microglobulin react with

surface structures in group A and certain group B, C and G strains. The binding of these proteins was interrelated but not related to binding of IgG (Kronvall et al., 1978; Kronvall et al., 1979b; Schönbeck et al., 1981). The reaction between fibrinogen and streptococci was originally described by Tillett & Garner (1934) and later ascribed to the M-protein (Kantor & Cole, 1959; Hryniewicz et al., 1972). Recently, group A and G streptococcal strains carrying the T4 antigen have been shown to interact with human haptoglobin of type 2-2 or 2-1, but not with 1-1 (Köhler & Prokop, 1978; Köhler et al., 1979). In addition, radiolabelled human albumin was shown to bind to groups C and G (but not group A) streptococci (Myhre & Kronvall, 1980d). In other test conditions, binding of human albumin to the lipid part of lipoteichoic acid was demonstrated (Simpson et al., 1980).

Purposes of the Study

The investigations were intended

- to study precipitation reactions between human IgG and IgA and streptococcal immunoglobulin receptors
- to study variations in IgG Fc-receptor specificity among certain streptococcal strains
- to localize the part of the IgA molecule that is bound by the streptococcal IgA receptor
- to study the interrelation between IgA and IgG receptors and their relations to other streptococcal cell wall components
- to isolate and characterize IgG Fc-binding material from type 15, group A streptococci
- to elucidate whether the immunoglobulin receptors might have any role in determining streptococcal virulence
- to study the opsonization of group A streptococci displaying IgG Fc-receptors

Present Investigations

A. DEMONSTRATION OF SOLUBILIZED STREPTOCOCCAL IgG AND IgA Fc-RECEPTORS

Whereas streptococcal immunoglobulin receptors have been studied mainly by using intact bacteria (see Introduction), papers I, II, III and V deal with the possible occurrence and Ig-binding properties of such receptors in streptococcal extracts. In the present investigations, classical Lancefield extraction at pH 2.0 was used at first (I, II); however, in subsequent work alkaline extraction at pH 10.0 has been preferred, as precipitates-in-gel formed by alkaline extracts were found to be more distinct than those formed under similar conditions by acid extracts. Corresponding results as regards M-protein, ascribable to particle size, have been reported by Fox (1974). Thus, large portions of M-protein are solubilized by alkaline extraction, while small, heterogeneous fragments are produced under acid conditions, creating the "multiple banding phenomenon" (Fox, 1968) in acrylamide gel electrophoresis. However, in the present work no major quantitative differences have been found as regards precipitating or agglutinating properties between the Ig-receptors, solubilized by acid vis-à-vis alkaline extraction. The introduction of protease inhibitors during solubilization of these receptors will be described later (Section B:2).

1. Agglutinating IgG Fc-Receptor in Extracts of Various Group A, C and G Streptococci

Sheep red blood cells coated with rabbit antibodies are agglutinated by high dilutions of staphylococcal protein A (Sjöquist & Stålenheim, 1969). In contrast, extracts of streptococci containing IgG Fc-receptors usually agglutinate at low titres only (Christensen & Kronvall, 1974; Havlíček, 1978). The agglutination caused by streptococcal extracts was shown by Havlíček (1978) to be mediated by the Fc-portion of rabbit IgG.

The modest titres noted for cells coated by rabbit IgG prompted an investigation with use of human red blood cells coated by "incomplete" human anti-Rh antibodies. It was found that Ripley-coated cells were agglutinated by streptococcal extracts at considerably higher dilutions than cells coated with other anti-Rh antibodies (II). It is of interest that the Ripley coat is exceptional among anti-Rh antibodies in being complement-binding and not limited to one subclass; it also detects rheumatoid factors more effectively than do other coats (Waller & Lawler,

1962). Only 7 out of 12 other anti-Rh antibodies coated for agglutination by type 15 extract (I), thus confirming the restriction in affinity for human IgG also demonstrated in precipitation experiments (see Section B:1).

When examined in five selected agglutination systems, human polyclonal IgG Fc was found inhibitory at 0.01 - 0.001 g/l, while IgG Fab, in concentrations up to 0.5 g/l was not (II). It was concluded that the agglutination of human IgG-coated cells by streptococcal extracts was caused by solubilized IgG Fc-receptors, in analogy to that with cells coated with rabbit IgG (Havliček, 1978). These experiments did not reveal any affinity of the extracts for IgG Fab-parts (cf. Section B:3).

In conclusion, human red blood cells sensitized with some "incomplete" anti-Rh antibodies are more sensitive than cells coated with rabbit IgG for the detection of streptococcal Fc-receptors; however, the Ripley anti-Rh is available in limited quantities only and other coats with similar advantages seem to be rare.

2. Precipitating IgG Fc-Receptor in Type 15 Group A Streptococci

In 1959, Wood & Schramm found that an acid extract of type 15 group A streptococci precipitated all of 137 human sera in capillary precipitin tubes. Although the serum factor taking part in precipitation was not characterized, the streptococcal factor involved seemed to resemble the M-protein in some respects, e.g. trypsin sensitivity.

By gel diffusion, the results of Wood & Schramm were essentially confirmed. Among the sera from 39 healthy Swedes, 82 % gave a single precipitation line against Lancefield extract of type 15 streptococci. A minority of the sera gave additional precipitates. The "main" precipitate did not occur with a trypsinized extract. Various experiments were performed to elucidate which serum component was responsible for the "main" precipitate. None of 21 sera with an IgG content < 7 g/l precipitated, whereas several sera with an IgG content > 7 g/l, but with low levels of IgA and IgM, could precipitate. Serum from a patient with agammaglobulinemia did not precipitate.

In another experiment, precipitates obtained in tubes from each of 8 normal sera and the type 15 extract were isolated by centrifugation and redissolved by trypsinization. When tested by double diffusion, all were precipitated by anti-IgG, but not by anti-IgA or anti-IgM. These experiments showed that IgG participated in the "main" precipitate against type 15 extract (I).

Commercial human IgG gave, as expected, the "main" precipitate, while IgG Fc or Fab did not (cf. Section B:3). In order to elucidate which part of IgG was reacting with the streptococcal factor, type 15 (alkaline) extract was run by electrophoresis at pH 8.6 and subsequently diffused against human sera (III). The precipitate corresponding to the "main" line was identified in

the isoelectric region. On addition of polyclonal IgG Fc (but not Fab) to the extract before electrophoresis, the streptococcal component eliciting this precipitate was moved to a more anodal position (III, Fig. 2). Thus, it was demonstrated that the "main" precipitate was caused by an IgG Fc-receptor.

The streptococcal IgG binding component was also localized by testing fractions obtained by preparative gel electrophoresis of crude type 15 extract, for agglutination of Ripley-coated human red cells. An agglutinating activity was exhibited only by the isoelectrically located fractions corresponding to the position of the IgG Fc-receptor precipitate. The mechanisms of precipitation and the restriction in capacity to precipitate normal sera will be further discussed (see Section B:3).

3. Separate IgA- and IgG-Receptors in Type 4 Group A Streptococci

Solubilization of the receptors

The presence of an IgA-receptor in certain group A streptococci was originally demonstrated by the binding of radiolabelled human IgA myeloma protein to intact bacteria; cross-inhibition experiments with IgA and IgG indicated that the receptors for these immunoglobulins on type 4 group A streptococci are distinct structures (Christensen & Oxelius, 1975)

In paper V, the possibility of solubilizing the IgA-receptor of type 4 group A streptococci was studied. An alkaline extract of type 4 (alkM4) was run by electrophoresis at pH 8.6 and the extract subsequently diffused against 27 normal Swedish sera. Eighty-five per cent of the sera gave a precipitate (3_{T4}), located slightly cathodal to the extract application well, whereas additional precipitates were created by a minority of the sera (V, Fig. 1). The position of 3_{T4} and the frequency with which the line was produced by normal sera closely resembled the results obtained with type 15 IgG Fc-receptor. Furthermore, individual sera displayed a concordance with regard to precipitation of type 15 Fc-receptor and eliciting 3_{T4} (unpublished observation). However, the type 4 component was apparently not an IgG Fc-receptor, since addition of IgG Fc, or sera containing IgG M-component, to the extract before electrophoresis did not affect 3_{T4} . On the other hand, each of 9 sera containing an IgA M-component caused anodal displacement of 3_{T4} (V, Fig. 1), the extent of displacement correlating with the electrophoretic mobility of the respective M-component (V, Fig. 3). Furthermore, IgA myeloma protein, purified from one of the sera, also caused an anodal displacement. The results showed that 3_{T4} was caused by a reaction with IgA not involving the antigen-combining sites. Obviously, the streptococcal component represented the IgA-receptor, earlier demonstrated on intact bacteria.

Like the majority of type 4 group A streptococci, the strain investigated displayed binding of both IgA and IgG. The presence of a solubilized IgG-receptor in alkM4 as well as acid

type 4 extract was shown by the capacity of both to agglutinate Ripley-coated human red cells (at a dilution of 1:1024). By examining fractions from preparative electrophoresis of alkM4 the position of the IgG-receptor was found to correspond to that of the IgA-receptor (V). Nevertheless, no precipitate caused by the type 4 IgG-receptor could be identified when investigating several normal human sera (V), though later experiments (unpublished data) demonstrated that polyclonal IgG at a concentration of 40 g/l precipitated the IgG Fc-receptor in alkM4. On electrophoretic separation, under conditions as in V, the position of this precipitate was similar to that of the IgA-receptor. It was evident that the IgG-receptor in alkM4 had a lower precipitating tendency than the IgA-receptor or type 15 IgG-receptor, similarly solubilized.

The Fc-part of IgA interacts with IgA-receptor

While the streptococcal IgG-receptors have been shown to interact with IgG Fc (Christensen et al., 1976a), the part of the IgA molecule involved in the binding to the IgA-receptor has not been determined previously. Recently, bacterial enzymes selectively cleaving human IgA1 into Fc- and Fab-fragments have been discovered. Such proteases have been found in several bacterial species, including *Streptococcus sanguis* (Plaut et al., 1974), *Neisseria meningitidis* and *Neisseria gonorrhoeae* (Plaut et al., 1975), *Streptococcus pneumoniae* and *Haemophilus influenzae* (Kilian et al., 1979). The reactivity of intact type 4 group A streptococci with IgA Fc and Fab produced by the use of such enzymes was investigated (IV).

In order to obtain Fab- and Fc-fragments, IgA1 myeloma protein was digested with gonococcal protease as described by Plaut et al. (1975). The digested IgA1, fractionated by preparatory gel electrophoresis, was tested for capacity to inhibit the binding of radiolabelled intact IgA1 to type 4 streptococci. The fractions containing IgA Fc inhibited the binding, whereas the Fab fractions did not (IV, Table 1). The same results were obtained with digested IgA, separated by gel filtration on Sephadex G200 - only the fractions containing IgA Fc blocked the binding of IgA1 to type 4 streptococci (IV, Fig. 3). When testing the pooled fractions containing IgA Fc or IgA Fab, only the former blocked the binding of IgA1 to type 4 streptococci; furthermore, the inhibitory capacity was approximately threefold (on a weight basis) that of undigested IgA1 (IV, Fig. 1) corresponding to equimolar binding activities of IgA and IgA Fc.

Similar results were obtained with IgA1 fractions obtained by digestion with *S. sanguis* protease, as shown in Table 1. The cleavage point of this protease is located eight amino acid residues towards the amino-terminal end of the chain, compared with that of *Neisseria* species (Plaut 1978). Hence, the eight amino acids are of no importance for the binding of IgA1 to type 4 group A streptococci.

Table 1. Uptake of radiolabelled IgA1 myeloma protein on whole type 4 group A streptococci: inhibition experiments with fractions from preparatory electrophoresis of IgA1 after digestion with *S. sanguis* protease ^{a)}

Fraction no.	Precipitated by ^{b)}		Uptake (%) of radiolabelled IgA on type 4 streptococci
	anti-IgA serum	anti-kappa serum	
2	(+)-	(+)-	74.1
3	(+)-	(+)-	71.6
4	(+)-	(+)-	73.1
5	(+)-	+	77.7
6	(+)-	+	64.1
7	++	+	3.4
8	++	-	7.4
9	++	-	7.4
10	++	-	17.7
11	-	-	74.1
12	-	-	72.8

a)The test was performed similar to the experiments described for digestion of IgA with gonococcal protease (paper IV).

b)Probably small amounts of undigested IgA2 in fractions 2-6.

Table 2. Prolonged incubation of M4 streptococci with labelled IgA1 myeloma protein; influence of benzamidine chloride ^{a)}

	Percentage labelled IgA1 bound to M4 streptococci on		
	day 1	day 2	day 3
M4 + PBS	71	45	22
M4 + benzamidine chloride	72	71	71

a)To 200 μ l standard suspension of M4 streptococci was added 10 μ l of benzamidine chloride (final concentration 0.04 mM) or PBS, and 50 μ l (2.5 μ g) of ¹²⁵I-labelled IgA1 myeloma protein and the mixtures incubated at 37°C. At 1, 24 and 48 h, the bacteria were pelleted by centrifugation and resuspended in 200 μ l PBS, or PBS containing benzamidine chloride. 100 μ l of the supernatants obtained was mixed with 200 μ l standard suspension of fresh M4 and the binding of radioactivity to the bacteria after 30 min incubation at 22°C determined.

It is concluded from these experiments that the binding of IgA1 myeloma protein to type 4 group A streptococci occurs through the Fc-portion of IgA. To the authors knowledge this is the first demonstration of a bacterial substance with such binding specificity.

Absence of IgA-specific protease activity

The usefulness of type 4, group A streptococci as a solid phase reagent for the determination of bacterial IgA-cleaving enzymes was recently demonstrated (Lindahl et al., 1981).

The binding of IgA to M4 streptococci might hypothetically occur to an IgA protease present in this particular type though absence of IgA-specific protease(s) in group A streptococci has been reported (Genco et al., 1975). Due to the binding of IgA Fc and intact IgA to the bacteria in this case, the proof of IgA-specific protease activity would apparently rest on the demonstration of free IgA Fab-fragments. Two experiments were performed to elucidate this possibility.

M4 streptococci, grown in 20 ml Todd-Hewitt broth, were washed and suspended in 1 ml PBS. Five mg of IgA1 myeloma protein were added and the mixture incubated overnight at 37°C. After removing the bacteria by centrifugation, the supernatant was tested by immunoelectrophoresis as described by Plaut et al. (1975). Only a precipitate at the position of the native IgA1 (no lines corresponding to IgA Fc or Fab) was observed, indicating that the binding of IgA to the bacteria did not cause cleavage of IgA.

In another approach, M4 streptococci were mixed with radiolabelled IgA1 myeloma protein. Following incubation for 3 days at 37°C and daily washing of the bacteria a gradually decreasing proportion of the radioactivity, originally bound, remained on the bacteria. The release of radioactivity was inhibited by presence of benzamidine chloride, demonstrating that streptococcal protease(s) can either destroy or liberate the IgA-receptor (Table 2). The supernatants, prepared as described above during prolonged incubation of M4 with IgA1, were tested for capacity to bind to fresh M4 streptococci. It was found that more than 90 % of the radioactivity of the three supernatants was bound, indicating absence of significant amounts of free IgA Fab-fragments.

Thus no IgA-cleaving enzymes were demonstrated in M4 streptococci. On the other hand, the IgA-receptor was found to be susceptible to protease(s) of the homologous strain.

B. DIFFERENTIAL REACTIVITY OF STREPTOCOCCAL Fc-RECEPTORS FOR HUMAN IgG. INFLUENCE OF SOLUBILIZATION CONDITIONS ON THE FINE SPECIFICITY AND PRECIPITATING PROPERTIES OF ONE RECEPTOR

Cell-bound streptococcal immunoglobulin receptors do not bind more than one class of immunoglobulins; group A strains binding both IgG and IgA exhibit separate receptors for these (Christensen & Oxelius, 1975; IV). All human subclasses of IgG and IgA bind to the respective receptors (see Introduction).

The specificity of solubilized streptococcal Ig-receptors was investigated in papers I, II and VI. In contrast to the results obtained with intact bacteria, some solubilized IgG Fc-receptor preparations showed a restricted affinity for human IgG Fc. However, it was found that the specificity of Fc-receptor preparations may change upon use of protease inhibitors during solubilization indicating that streptococcal protease(s) can modify the receptors in this respect.

1. Restricted Affinity for IgG Fc of Some Streptococcal Receptors Solubilized without Presence of Protease Inhibitors

Strain variation in the affinity for IgG Fc

Lancefield extracts of group A streptococci, representing various types, as well as a group C and a group G strain were tested for IgG Fc-receptor activity, using human red blood cells coated with the Ripley, two IgG1 and one IgG3 anti-Rh, respectively (II). The extracts of 12 of 17 group A strains and of both the group C and group G strain agglutinated one or more of the coated cells. Generally, the titres for Ripley-coated cells were considerably higher than for the other coated cells.

According to their ability to agglutinate IgG1- and/or IgG3-coated cells, a diversification of the IgG-specificity of different strains was found. Thus, the extracts of three group A types (M4, T28 and M56) agglutinated IgG1- but not IgG3-coated cells, while the reverse was found with another extract (T44). By also including hemagglutination inhibition tests with isolated myeloma proteins, at least four different patterns of IgG specificity were identified, distinguished by the relative affinities for human IgG subclasses. Four IgG3 proteins, all Gm(+5), were inhibitory in only two out of five selected hemagglutination systems, while myeloma proteins of the other subclasses, with one exception, were inhibitory in all five systems. Hence the main restriction of solubilized streptococcal IgG Fc-receptors seems to be related to the IgG3 subclass. It is so far not known whether the IgG Fc-receptors of various streptococci can exhibit allotype-related specificities, as was found with type 15 (see below). However, the differences reported here showed that the streptococcal Fc-receptors are not uniform in their specificity for human IgG Fc.

Allotype specificity of Fc-receptor from type 15 group A streptococci, solubilized in absence of protease inhibitors

The sera from various ethnically homogeneous populations were tested in immunodiffusion for capacity to precipitate type 15 acid-extracted IgG Fc-receptor. A pronounced geographical variation in this capacity was found (I). As the frequencies of the IgG allotype markers G1m(4) and G3m(5) vary markedly in these populations (see Grubb, 1970), the possible preferential activity of the Fc-receptor for certain markers was studied. Of the 32 normal Swedish sera precipitating the Fc-receptor (I), 94% were Gm(+5). Originally, seven non-precipitating sera were found (I), and another 16 were identified later. Of the 23 non-precipitating sera, 70% were Gm(+4, +5), 26% Gm(-4,-5) and one Gm(-4,+5) (Table 3). Thus, the capacity to precipitate correlated significantly to presence of Gm(5) ($P < 0.05$).

Table 3. Occurrence of the IgG allotype markers G1m(4) and G3m(5) among normal Swedish sera precipitating and not precipitating acid-extracted IgG Fc-receptor of type 15 group A streptococci in plain agarose gel

Sera	Gm(+5)	Gm(-5) ^{a)}
Precipitating ^{b)} (N = 32)	30 (94 %)	2(6 %)
Non-precipitating (N = 23)	17 (74 %)	6(26 %)

a) A significantly higher proportion of precipitating vis-à-vis non-precipitating sera belonged to Gm(+5) ($p < 0.05$; Chi-square test, one tail).

b) A minority of sera giving weak lines were classified as precipitating.

However, the finding (Table 3) that 74% of the normal sera not precipitating type 15 extract were Gm(+5) clearly showed that factors other than the presence of this marker were also important for precipitation to occur. The human sera were not bimodally distributed with respect to capacity to precipitate the Fc-receptor. While the majority of precipitating sera gave a distinct precipitate even when diluted eight-fold certain sera only precipitated undiluted. It is possible that more than one IgG precipitating substance was present in the crude type 15 extract or that certain IgG molecules participated in the formation of precipitate by the Fab parts (cf. Section B:3).

All these experiments were performed using plain agarose gel. Other, differing results were found in later experiments (unpublished) using high-molecular gel, viz. an agarose gel supplemented with 10% (w/v) Dextran T10 (prepared as in VI). When testing normal sera against type 15 (alkaline) extract several precipitated at high dilutions. For example, one serum (KP) precipitating at a dilution of 1:32 in plain agarose gel could precipitate at a dilution of 1:128 in the high-molecular gel. Another serum (IE) not precipitating type 15 extract in plain agarose gel could precipitate at a dilution of 1:128 in the high-molecular gel. Thus, normal human sera did not segregate with respect to precipitation of type 15 Fc-receptor in the high-molecular gel.

The IgG Fc-receptor present in type 15 alkaline extract (prepared in the absence of protease inhibitors) was partially purified by passing the extract on an immunosorbent column of IgG3(5) myeloma protein coupled to Sepharose 4B. The IgG-binding substance attached and eluted agglutinated IgG1-coated human red cells at a dilution of 1:100, whereas IgG3-coated cells were agglutinated at a higher dilution, 1:6,400. Of two IgG3 myeloma proteins, a Gm(+5) protein showed a ten-fold higher inhibitory capacity in the latter system as compared with a Gm(-5) protein. Similarly, this IgG-binding substance precipitated the Gm(+5) but not the Gm(-5) protein (both at 2.0 g/l) in high-molecular gel. The preference for Gm(+5) of this receptor was thus in agreement with the immunodiffusion experiments in plain agarose gel with normal human sera. IgG1, 2 and 4 proteins (2 g/l) were found to precipitate the purified receptor without apparent restriction (unpublished data).

To sum up, type 15 IgG Fc-receptor, solubilized in the absence of protease inhibitors, displayed an allotype-related restriction in the affinity for human IgG3, while the affinity for the other human IgG subclasses was unrestricted. The IgG determinant detected by this Fc-receptor appears thus to be of an iso-allotypic character. During subsequent attempts to characterize this Fc-receptor, it was found that the activity of various solutions often decayed rapidly, which made the isolation of the substance hazardous. These aspects will be discussed in the following section.

2. Unrestricted Affinity for IgG of Fc-Receptor from Type 15 Group A Streptococci Prepared in Presence of Protease Inhibitors

Repeated observations in our laboratory have indicated that solubilized streptococcal Ig-receptors often deteriorate during storage or isolation experiments. As the receptors are sensitive to trypsin (III, V), it was thought that bacterial proteases (Elliott, 1945) might account for these changes. The presence of proteolytic activity in type 15 streptococcal alkaline extract was readily demonstrated by digestion of casein (unpublished observation). In order to neutralize such enzyme activity, the solubilization of Ig-receptors was modified by including four different protease inhibitors (VI).

Type 15 alkaline extract prepared in the presence of protease inhibitors (alkM15-i) precipitated both the normal sera KP and IE at a serum dilution of 1:32 when examined in plain agarose gel (Table 4); in addition, each of four other sera which, like IE, did not precipitate type 15 extract, when prepared in the absence of protease inhibitors (alkM15), showed an equal capacity to precipitate alkM15-i. Similarly, no significant differences between various normal sera were observed when using high-molecular (dextran containing) gel. In this gel, normal sera gave visible precipitates with the receptor at dilutions between 1:128 and 1:512. Thus, alkM15-i showed no restriction as regards precipitation of normal human sera.

AlkM15-i precipitated monoclonal IgG at ten-fold higher dilutions than did alkM15. Thus, in the high-molecular gel, alkM15-i precipitated myeloma proteins at a concentration of 0.2 g/l. Furthermore, there was apparently no restriction in the affinity for IgG of the Fc-receptor of alkM15-i, as an IgG3 Gm(-5) protein also precipitated. Yet another difference between alkM15 and alkM15-i was that only the latter precipitated IgG Fc-fragments, a finding that will be discussed below.

Table 4. Precipitating dilutions of two normal human sera tested in plain agarose gel by double radial diffusion against crude, alkaline extract of type 15 group A streptococci, prepared in absence (alkM15) or presence (alkM15-i) of protease inhibitors

Serum	Precipitating serum dilution	
	alkM15	alkM15-i
KP	1:32	1:32
IE	No precipitate when undiluted	1:32

Similar to alkM15-i, the Fc-binding substance (0.2 g/l), isolated from this extract precipitated all examined IgG myeloma proteins as well as polyclonal IgG Fc (VI). These results indicate that type 15 IgG Fc-receptor, solubilized in the presence of protease inhibitors, is unrestricted as regards affinity for human IgG Fc in contrast to type 15 Fc-receptor, solubilized without presence of inhibitors, which showed a restriction in the affinity for IgG3 Fc. Conceivably, streptococcal protease(s) may modulate type 15 Fc-receptors, "originally" unrestricted, to be restricted in their affinity for IgG3 Fc. However, the possibility that streptococcal protease(s) might induce conformational changes in some IgG molecules remains to be studied. Alternatively, these findings might simply be attributable to differences in the polymerization tendency (cf. VI) of soluble Fc-receptors depending on the use of protease inhibitors; some support for this theory was provided by the finding that alkM15-i and the substance purified therefrom, (but not alkM15) had the capacity to precipitate IgG Fc, which requires a minimum of two Fc-binding sites.

3. Mechanism of Precipitation of Immunoglobulins by Streptococcal Fc-Receptors - Participation of Fab-Regions?

Immunoelectrophoresis experiments (III) showed that the precipitate representing reaction between normal human sera and solubilized IgG Fc-receptor in type 15 extract (alkM15; protease inhibitors not used) was more anodally positioned on addition of IgG Fc to the extract before electrophoresis. The fact that this precipitate was not suppressed, but was changed as regards electrophoretic position, was in agreement with Ouchterlony tests showing no precipitate formation in plain agarose gel between IgG Fc and alkM15. Thus, an additional serum factor was apparently required for precipitation of complexes of Fc-receptor and IgG Fc; furthermore, the finding that isolated polyclonal IgG precipitated the receptor (III) strongly indicated that this serum factor was IgG.

The character of the IgG molecules required for precipitate formation is not yet elucidated. Theoretically, they might represent conventional antibodies directed against the Fc-receptor, IgG Fc, or complexes of the two. So far, antibodies directed against streptococcal Fc-receptors have not been demonstrated. The second possibility seems less likely, as anti-IgG Fc does not occur regularly in normal human sera (see Grubb, 1970). The third kind of antibody mentioned would be directed against determinants revealed by the reaction between Fc-receptor and IgG Fc. Such antibodies, i.e. those specific for "hidden IgG-determinants", appearing in the complex of protein A and IgG Fc, have been claimed to occur in hyperimmunized animals (Lind, 1974; Lind & Mansa, 1974) and in normal human sera (Lind et al., 1975).

Alternatively, other streptococcal components, e.g. peptidoglycan or lipoteichoic acid might complex with the Fc-receptor, and antibodies directed against such components could participate in the precipitation. Anti-peptidoglycan antibodies, though frequently occurring in normal human sera (Schachenmayr et al., 1975; Schalén & Christensen, 1979), did not seem to be involved, as no interference was found between the precipitates due to peptidoglycan and Fc-receptor (III). Most normal sera also contain antibodies to teichoic acids (Martin et al., 1965). The experiments in (III) did not exclude complexes between Fc-receptor and lipoteichoic acid in alkM15; however, purified Fc-receptor was not significantly contaminated with lipoteichoic acid (VI).

According to another hypothesis, binding to IgG Fab-regions outside the antigen-combining sites might under certain circumstances be necessary for precipitation of IgG by streptococcal immunoglobulin receptors. A similar requirement for precipitate formation between staphylococcal protein A and IgG has been reported (Inganäs & Johansson, 1981).

The possibility that streptococci might bind IgG Fab was tested in uptake experiments using intact bacteria. Type 15 streptococci did not bind significant amounts of radiolabelled, monomeric IgG1 Fab; in contrast, a high binding capacity for artificial aggregates of IgG1 Fab was found. Interestingly, several group A strains displayed significant binding of aggregated (but not monomeric) IgG1 Fab and/or light chains. There was no correlation in the individual strains between occurrence of IgG Fc-receptor activity and capacity to bind such aggregates (manuscript in preparation). It remains to be elucidated, however, whether in certain strains, e.g. type 15, the capacity to react with Fab might contribute to precipitate formation with IgG, as was postulated for protein A. In analogy to type 15 IgG Fc-receptor, the precipitate between type 4 IgA-receptor and normal human serum was displaced anodally by addition of monoclonal IgA to alkM4 before electrophoresis (V), indicating "additional" serum components to be involved in precipitate formation. Since the sera precipitating the IgG Fc-receptor in alkM15 also precipitated the IgA Fc-receptor in alkM4 (unpublished observation) it is conceivable that Ig-determinants other than IgA Fc participated in the precipitation. It should be mentioned that the M4 strain also bound aggregated IgG Fab.

As stated above, the presence of, protease inhibitors during extraction of type 15 IgG Fc-receptor resulted in enhanced precipitating capacity. The findings that both sera at very high dilutions and IgG Fc (VI) precipitated might conceivably be attributable to the physical state of this Fc-receptor; thus, marked elongation and/or polymerization of the purified IgG-binding material was demonstrated (VI). Similarly, the Ig-receptors of type M4, solubilized in the presence of protease inhibitors, displayed considerably higher precipitating capacity vis-à-vis those prepared without presence of inhibitors.

C. FURTHER CHARACTERIZATION OF STREPTOCOCCAL IMMUNOGLOBULIN RECEPTORS. RELATION TO OTHER STREPTOCOCCAL COMPONENTS AND TO STAPHYLOCOCCAL PROTEIN A

In papers III and V, the relationships between solubilized immunoglobulin receptors of type 15 and type 4 group A streptococci, respectively, vis-à-vis other streptococcal components were investigated by electrophoretic methods. The Ig-receptors were found to be distinct from several known streptococcal antigens. In paper VI, the successful isolation of an IgG Fc-binding substance from type 15 streptococci is described and data on its chemical composition given.

1. Immuno-electrophoretic Analysis of Crude Streptococcal Extracts

On diffusion of electrophoretically separated type 15 alkaline extract against normal human sera or rabbit antistreptococcal sera, four distinct precipitation systems were identified (III, Fig. 1). An anodally positioned precipitate was formed by reaction with an unknown streptococcal component, sensitive to trypsin. This component was not type-specific, as type 4 extract gave an identical line. A precipitate representing the IgG Fc-receptor was localized to the isoelectric region at the pH value used, 8.6. It was separate from the lines caused by reactions with peptidoglycan and group-specific carbohydrate, both also slowly migrating. Due to different electrophoretic mobilities, the Fc-receptor and the type 15 M-antigen were clearly separable.

Analogous immuno-electrophoretic analysis of type 4 alkaline extract revealed four distinct lines, caused by the anodal component, peptidoglycan, IgA-receptor, and an additional component of unknown origin, respectively (V, Fig. 1). As the last-mentioned precipitate, 4T₄, was abolished by trypsin treatment of the extract, it might have been elicited by type 4 M-antigen - however, the line was not obtained with the available rabbit anti-M₄ serum. As earlier discussed (see Section A:3), an IgG Fc-receptor was localized to the same region as the IgA-receptor by testing fractions obtained by preparatory electrophoresis of the extract, for agglutination of IgG-coated red blood cells. This receptor did not precipitate human sera under the conditions used. Purified haptoglobin did not interfere with any of the precipitates or with the agglutination of IgG-coated cells; thus, the haptoglobin-binding structure, allegedly identical with T4 protein (Köhler & Prokop, 1978), was distinct from the immunoglobulin receptors. The lipoteichoic acid was shown to have a very wide electrophoretic mobility on the basis of tests for red cell sensitizing capacity. Thus, the immunoglobulin receptors were distinct from

lipoteichoic acid, as also demonstrated by purification of type 15 IgG Fc-receptor (see below).

2. Purification of a Streptococcal IgG Fc-Binding Substance

The isolation of an IgG Fc-binding substance from type 15 group A streptococci is described in paper VI. It was found that the use of protease inhibitors during the solubilization and purification procedures was a crucial prerequisite for recovery of the Ig-binding substance in a stable form.

On the assumption that the decay was attributable to streptococcal protease activity (see Elliott, 1945), various enzyme inhibitors were introduced during solubilization and isolation of IgG Fc-binding material. Benzamidine chloride and iodoacetic acid were added to the broth cultures, to washings and to all buffers subsequently used. In addition, diisopropylfluorophosphate (DIFP) and phenylmethylsulfonyl-fluoride (PMSF) were added to the alkaline extract immediately before extraction and following removal of the extracted bacteria. The crude type 15 extract was run on a DEAE cellulose column and IgG-binding fractions pooled. In the second purification step, affinity chromatography on a column of human, polyclonal IgG bound to Sepharose was used; IgG-binding activity was eluted with Tris buffer containing 0.5 M NaCl and 0.1 M sodium acetate, pH 3.5.

As with protein A of *Staphylococcus aureus* (Kronvall, 1973b; Sjöquist et al., 1972), but in contrast to M-protein (Havlicek 1975) and T-protein (Ludwicka & Kloczewiak, 1978), the IgG-binding material could not be traced during the isolation procedure by UV light extinction at 280 nm, indicating a low content of aromatic amino acids; however, the substance was easily detected by its IgG-binding activity, e.g. by precipitation or agglutination tests.

The molecular weight of the purified substance was estimated to approximately 29,500 by SDS-polyacrylamide gel electrophoresis. As determined by gel filtration the IgG-binding substance exhibited a hydrodynamic volume close to that of IgA, showing an elongated shape, or a tendency to polymerization. Peptidoglycan was absent from the purified preparation, as no glucosamine was detected; furthermore, the possible contamination of lipoteichoic acid, being retained on DEAE, was minimized by the present procedure.

The IgG-precipitating capacity of the purified material was estimated by single radial diffusion in a gel containing known amounts of monoclonal IgG. It was found that 1 mg of the substance precipitated 0.6 - 0.7 mg of IgG. As this ratio corresponds to only 0.10 moles of IgG precipitated by one mole of the IgG-binding material the data seem to give further

confirmation of the marked propensity of the Fc-receptor to form polymers, conceivably a prerequisite for precipitation of IgG. Similar to crude alkaline type 15 extract, prepared in presence of protease inhibitors (Section B:2) the purified substance precipitated human polyclonal IgG Fc and monoclonal IgG (but not IgA, IgM or IgD) in double immunodiffusion.

Christensen & Holm (1976) reported on purification of IgG Fc-receptor of group C streptococci, solubilized by phage-associated lysin; protease inhibitors were not used during the isolation procedure. The molecular weight found, 60,000, closely corresponds to a dimer of type 15 group A streptococcal IgG-binding substance. Interestingly, solubilization of M-protein by phage-associated lysin was shown also to result in larger molecules as compared to other methods (Phillips et al., 1981).

There is no method available to quantitate the amount of Fc-receptors in the cell wall of intact organisms. By using single radial diffusion in a gel containing monoclonal IgG for quantitation of soluble Fc-receptor, it was found that 11 % of the IgG-binding material present in the crude alkaline extract had been recovered in the purified preparation, and that the purification degree was 25-fold. In later experiments, using freshly prepared IgG-immunosorbents, however, virtually all Fc-binding material was eluted with the acetate buffer, providing higher yields than reported in paper VI. Furthermore, preliminary experiments have indicated that the yield of Fc-binding substance by extraction of bacteria using detergents may be higher than that obtained by alkaline hydrolysis.

3. Immunoglobulin Receptors as Distinct Constituents of Streptococci

Earlier knowledge of the relation between the streptococcal IgG Fc-receptors and other cell wall components was based mainly on studies on intact bacteria. The relative heat resistance of the Fc-receptors showed that they were not identical with various enzymes, streptolysins or T-antigen, and blocking experiments ruled out any possible connection with certain heat-stable enzymes, viz. deoxyribonuclease and streptokinase (Christensen & Kronvall, 1974; Christensen, 1976).

It emerged from the electrophoretic studies (III; V) that solubilized immunoglobulin receptors from types 4 and 15 group A streptococci are distinct from several cell wall components, viz. peptidoglycan, group-specific carbohydrate, lipoteichoic acid or the haptoglobin binding structure of type 4, allegedly identical with T4 protein. The results were not surprising as these components are resistant to trypsin, in contrast to the Ig-receptors. However, as M-protein is sensitive to trypsin (Lancefield, 1962) it was of special interest to note that type 15 M-protein and IgG Fc-receptor were clearly separable with

respect to electrophoretic mobility (III) and that the IgG Fc-binding substance, isolated from the type 15 strain, did not precipitate anti-M15 sera (VI). The results were in agreement with those of Havlíček (1978) who was able to separate acid-extracted M8 protein and IgG Fc-receptor by gel-filtration on Sephadex G200. Recently, the separation of phage-associated lysin-extracted M-protein and IgG Fc-receptor of types 1 and 12 was also reported (Kühnemund et al., 1981). Thus, there are no available data suggesting that M-proteins and Ig-receptors may be identical. On the other hand, it seems quite probable that many conventionally prepared, acid-extracted M-proteins may have been contaminated by Ig-receptors co-purified by such methods. For instance, precipitation with 30-40 % ammonium sulphate followed by gel filtration and/or ion-exchange chromatography for fractionation of acid extracts of types T28, T44 and M55 group A streptococci, did not separate the IgG Fc-receptors from the OF antigens (Schalén et al., 1979), allegedly closely associated with M-protein (Maxted & Widdowson, 1972). The proposition of Kühnemund et al. (1981) that an IgG-immunsorbent step should be included in procedures for M-protein purification therefore deserves attention.

Chemical analysis of purified type 15 IgG Fc-receptor showed the substance to be a protein, distinguished by a high content of glutamic acid and alanine and low content of tyrosine, proline and phenylalanine (VI). Although type 15 M-protein has not been analysed chemically, the composition of the Fc-receptor reveals certain similarities with M-proteins of type 24 (Beachey et al., 1978) and type 6 (Fischetti et al., 1976). It is not yet known whether sequence homologies with the M-proteins may exist.

The M-associated proteins (MAP; Widdowson et al., 1971) constitute a less well defined entity; they have still not been purified. MAP, also designated as non-type specific antigens associated with M-proteins (Beachey et al., 1973; Vosti, 1975), are trypsin-sensitive proteins that co-purify with M-protein by conventional procedures. They are divided into at least two serologic types, of which type I is more antigenic than type II (Widdowson et al., 1976; Widdowson, 1980). The pathogenic role, if any, of MAP is unknown. Elevated levels of antibodies to MAP, mainly of the IgG3 subclass are observed in rheumatic fever (Mortimer & Widdowson, 1979). Although the Ig-receptors have not been proved antigenic, it seems clear that they would fulfil the criteria for MAP, being closely associated with M-proteins, but not type-specific.

An indication that MAP and Fc-receptors are not generally identical emerges from the fact that MAP is present in some group A streptococcal types which seem not to produce Fc-receptors, e.g. M12 and M30. Thus, it was recently found (Christensen et al., in press) that partially purified MAP of type 30 did not contain IgG Fc-receptor activity. However, in

type 15 alkaline extract, prepared without the use of protease inhibitors, MAP and Fc-receptor had the same electrophoretic mobility. Furthermore, there was a correlation between anti-MAP titres of normal human sera and capacity to precipitate this Fc-receptor in plain agarose gel, suggesting that MAP and Fc-receptor of type 15 were identical. On the other hand, type 15 IgG Fc-binding material, solubilized and purified in the presence of protease inhibitors (VI), was devoid of MAP activity. The interpretation of this finding is not clear, as the high Fc-binding activity of this preparation might disturb the test for MAP activity, based on complement fixation (Widdowson et al., 1971). Obviously, the elucidation of these relations will require other detection methods for MAP.

It is concluded that the IgG- and IgA Fc-receptors of group A streptococci are cell wall proteins readily separated from various constituents (including some M-proteins) hitherto investigated. At present, however, it is not known whether some of the Fc-receptors might be identical with MAP. As MAP are of at least two principal types, the possibility that some of the MAP are identical with Fab-binding structures should also be studied.

4. Discussion of Cellular Localization of Immunoglobulin Receptors

According to current thinking, peptidoglycan constitutes the matrix of the streptococcal cell wall, extending from the protoplast membrane to the bacterial surface (Wagner et al., 1978). Due to its close linkage to the group-specific carbohydrate, the peptidoglycan is markedly resistant to biodegradation (Smialowicz & Schwab 1977) and it is also held to be of crucial importance for streptococcal pathogenicity (Heymer, 1975; Cromartie et al., 1977).

On the other hand, both resistance to phagocytosis (Fischetti et al., 1976) and adherence to epithelial cells (Ellen & Gibbons, 1972) are determined in virulent streptococci by villous surface protrusions. The localization of M-protein to these projections has been demonstrated by electron microscopy (Cole, 1968; Swanson et al., 1969). The finding that the surface projections can be removed by non-ionic detergent treatment showed that these are formed by non-covalent interactions (Fischetti et al., 1976). A complex formation between M-protein and the negatively charged polyglycerophosphate moiety of lipoteichoic acid was recently demonstrated (Beachey, 1981). Thus, while contact with epithelial cells seems to be mediated by the fatty acid residue of lipoteichoic acid (Ofek et al., 1975) M-protein is also allegedly important for adhesion (Ellen & Gibbons, 1972; Grabovskaya et al., 1980).

Electron microscopic studies have revealed that the IgG Fc-

receptors are located at the filamentous protrusions of group A streptococci (Christensen et al., 1976b; Ryć et al., 1981). The way in which the Fc-receptors are linked to the streptococcal cell wall is not known. The recent finding (Ginsburg et al., in press) that certain polyelectrolytes react with lipoteichoic acid (but not with purified IgG Fc-receptor), yet are capable of blocking the binding of IgG to streptococcal Fc-receptors, may indicate that the receptors are attached to lipoteichoic acid. Covalent binding of staphylococcal protein A to peptidoglycan has been demonstrated (Sjöquist et al., 1972). The probable linkage between the Ig-receptors and other cell wall components remains to be investigated.

By absorbing sera with streptococci, significant amounts of IgG may be non-specifically bound to Fc-receptors (Christensen et al., 1977a). In this way, the binding of IgG to surface Fc-receptors would conceivably account for some of the previous results, based on absorption studies and interpreted as cross-reactions between M-types (Fox & Wittner, 1968; Wiley & Bruno, 1972) or cross-reactions between mammalian tissue and streptococci (for review see Christensen et al., 1979). Similarly, when demonstrating M-protein by immunofluorescence the non-specific binding of IgG to Fc-receptors should be borne in mind. Evidence will be presented that the Fc-receptors are of importance for streptococcal virulence (Section D). Conceivably, the antiphagocytic action of M-protein and IgG-receptors is facilitated by the localization of these proteins to the filamentous protrusions. As the M-protein and IgG Fc-receptor of some types have been demonstrated by immunochemical methods to be separate structures (see above), it is clear that confusion may arise if the presence of M-protein is defined by antiphagocytic property or by the demonstration of villous protrusions, as is common in the literature on streptococci.

5. Differing Properties of Fc-Receptor of Type 15 Group A Streptococci and Staphylococcal Protein A

The molecular weight of staphylococcal protein A, 42,000 (Björk et al., 1972) is greater than that of type 15 streptococcal IgG Fc-binding substance, 29,500 (VI). The amino acid composition of protein A (Sjöquist et al., 1972; Sjödahl, 1977b) differs in some respects from that of the streptococcal substance. Thus, protein A exhibits a higher content of aspartic acid and proline but a lower content of alanine, leucine and threonine, as compared with the streptococcal material. However, both display a high content of glutamic acid and lysine. Future studies will show whether streptococcal Fc-receptors similar to protein A (Hjelm et al., 1975; Sjödahl, 1977a) are composed of repetitive sequences.

Some data on the specificity for human IgG of protein A were mentioned earlier. Like protein A (Kronvall & Williams, 1969),

type 15 streptococcal IgG Fc-receptor, whether solubilized in the presence or absence of protease inhibitors, displayed an apparently unrestricted binding of the Fc-parts of human IgG1, 2 and 4 (II). On the other hand, it is evident that the substances differ with regard to reactivity with human IgG3 Fc. While protein A reacts with some IgG3 proteins, rarely occurring in Caucasians (Ito et al., 1980), the streptococcal Fc-receptor, cell-bound (Christensen & Oxelius, 1974; Myhre & Kronvall, 1980b) or solubilized in presence of protease inhibitors (VI) showed a wide, if not unrestricted, affinity for IgG3 Fc. Furthermore, type 15 Fc-receptor, solubilized in the absence of protease inhibitors, reacts with at least G3m(5) proteins, thus contrasting with protein A. The probable affinity of both substances for Fab determinants (see Section B:3) remains to be investigated in detail.

The segregation of human IgG1, 2 and 4 myeloma proteins on the basis of precipitability with protein A (Kronvall & Williams, 1969) was recently ascribed to the reactivity with IgG Fab, allegedly restricted (Inganäs & Johansson, 1981). Thus, protein A did not precipitate IgG Fc-fragments (Kronvall & Williams, 1971) thereby distinguished from type 15 streptococcal Fc-receptor, prepared in presence of protease inhibitors. On the other hand, type 15 Fc-receptor, prepared in the absence of inhibitors, did not precipitate IgG Fc.

To sum up - the chemical composition of staphylococcal protein A is different from that of type 15 streptococcal IgG Fc-receptor, though both are polypeptides. Whether any sequence homologies between these substances may exist is not yet known. Both protein A and type 15 streptococcal Fc-receptor are capable of forming precipitates with normal human sera and polyclonal IgG, conceivably due to an extended shape and/or polymerization. The IgG binding properties of protein A have not been shown (like type 15 Fc-receptor) to be affected by the use of protease inhibitors during solubilization. It is thus obvious that the streptococcal Fc-receptor, whether or not solubilized in the presence of protease inhibitors, differs from protein A with regard to affinity for the Fc-part of human IgG3.

D. STUDIES ON THE IMPORTANCE OF IgG Fc-RECEPTORS IN STREPTOCOCCAL VIRULENCE

Mouse-passaging has been the method of choice to increase the virulence of group A streptococci. In this procedure, mice are given a lethal dose of the bacteria intraperitoneally. After the animal has succumbed from septicemia, a suspension of the spleen (or heart blood) is injected intraperitoneally in another mouse. It is generally held that the streptococcal variants selected by mouse-passage produce greater amounts of M-protein than does the original strain (Todd & Lancefield, 1928; Lancefield, 1962). However, Burova (1979) showed that the streptococci obtained by mouse-passage may be devoid of the original M-protein. In paper VII, Fc-receptors were demonstrated in such highly virulent variants, in contrast to the original strains, indicating the possible importance of the receptors for streptococcal virulence.

1. Observations on Variants of a Type 12 Group A Streptococcal Strain Obtained during Serial Mouse-Passage

A type 12 group A streptococcal strain was serially passaged in mice 25 times (VII); the strain isolated from each passage was designated according to the number of passages performed prior to isolation. As expected, an increased virulence for mice, was recorded during the passages: LD₁₀₀ of M12/4 was 10^3 CFU, as compared with LD₁₀₀ of 10^8 of the original strain (VII, Fig.1). With the exception of strains M12/4-13, the ability to survive in human blood (growth index) remained high during the passages. Indirect bactericidal tests with rabbit anti-M12 serum (raised against the original strain) showed a strong bactericidal action against M12/1-3 but no effect on M12/14-25. On the other hand, M12/14-25 were opsonized by antiserum raised against M12/25 as well as against some other serially mouse-passaged strains (see Section E).

Lancefield extracts were tested for presence of M12 antigen by double diffusion against anti-type 12 serum (VII). Each of the extracts of M12/1-3 gave a precipitate showing complete fusion with the line given by the extract of M12. Such a precipitate was not obtained with any of the extracts of M12/4-25. In agreement with these findings, quantitation of extracts by electroimmunoassay (Eliasson et al., 1980) showed a gradual increase in M12 antigen in M12/1-3, whereas no M12 antigen was detectable in any of the extracts of M12/4-25 (VII, Fig.2). Nevertheless, the finding that variant M12/25 contained the T12 antigen showed that T12 was retained during the passages.

Hemagglutination testing of the acid extracts showed no IgG Fc-receptor activity in M12 or M12/1-3. However, such activity did appear in M12/5, increasing during subsequent passages and stabilizing at a high level from M12/12 (VII, Table 2).

2. Discussion of Changes Observed during Mouse-Passage of Type 3, 12 and 46 Group A Streptococci

As mentioned above, the virulence for mice, originally low, increased during the initial four passages of M12, the maximum level being reached after 7 passages (VII). Both the original strain and the mouse-passaged variants resisted phagocytosis in normal human blood. The M12 antigen (determined immunochemically and by indirect bactericidal tests) disappeared abruptly at the fourth passage and did not reappear in later passages. In contrast, IgG Fc-receptor activity, not present in the original strain, was noted in the variants obtained from the fifth and subsequent passages.

Two other strains, M3 and M46, were also subjected to serial mouse passages. Like M12, the strains lost the original M-antigens and acquired IgG Fc-receptor activity (II). These variants, 3P and 46P, as well as 12P(M12/25) were opsonized by rabbit antisera raised against each of them, viz. in homologous as well as in heterologous systems. Furthermore, both of two tested rabbit sera raised against M1 were opsonic and extracts of the strains were precipitated by these anti-M1 sera, giving lines in Ouchterlony-analysis fusing with that of the extract of a M1 reference strain. Taken together with the inhibition of strain 12P in the bactericidal test produced by anti-M1 serum (VII) the P-strains should thus be classified as belonging to type M1, following conventional typing procedures. Accordingly, 3P and 46P were found by Dr.G.Colman, Colindale, London, to be M1 strains (12P was not M-typable).

A simplistic interpretation of these data would be that the original M-strains were contaminated with type M1 group A streptococci, i.e. the patients from whom they were isolated had in fact been infected by two group A strains. However, it seems less likely, although not impossible, that all three strains M3, M12 and M46 were mixed with M1 streptococci. Also, contamination during the experiments should be considered as an explanation. This possibility was ruled out by repeating the passages with the type 12 strain on three different occasions (unpublished observation). An important clue is, however, that the results obtained (VII) apparently argue against both theories, as the strain underwent three phases during the passaging. Thus, M12/1-3 were M-type 12 by immunochemical and opsonic tests, whereas M12/14-25 were M1 strains (although M12/25 was still T-type 12). However, M12/4-13 belonged to neither of these types, as tested by precipitation (unpublished as regards M1) or bactericidal tests (Havliček, personal communication). A theory that the original M12 strain was a mixture of three strains would then necessarily have to account for the finding that M1 was not selected until the second strain (represented by M12/4-13) had

been selected and again disappeared.

It is important to note that M-proteins of group A streptococci are not stable structures. Since the early works of Lancefield it has been repeatedly recognized that M protein is readily lost by subculturing strains on artificial media. The genetic regulation of streptococcal M protein and other virulence factors has only recently been studied. Cleary et al. (1975) proposed that the synthesis of M-protein and opacity factor is under extrachromosomal control. The structural genes coding for M-protein and opacity factor were found to be separate although closely linked (Cleary, 1978). Transduction of genes determining M-protein and opacity factor production has been demonstrated by Totalian (1979) and Spanier & Cleary (1980).

In recent conjugation experiments, (Ravdonikas et al., in manuscript), triggering of IgG and IgA Fc-receptor as well as opacity factor production was obtained with plasmids determining erythromycin resistance. Group A, type 12, or group H streptococci were used as donors and tetracycline-resistant variants derived from a type M22, group A strain (devoid of M22 antigen) as recipients. In contrast to the parent strains, the transconjugants displayed IgG and IgA Fc-receptors, lack of typability, and antiphagocytic activity. The possibility of contamination was excluded as the transconjugants were chromosomally resistant to erythromycin and tetracycline. The fact that no extrachromosomal DNA was detected in the transconjugant, although it was erythromycin resistant, indicated that plasmid DNA was inserted into the chromosomal DNA.

The conclusive evidence that plasmids may also trigger the production of IgG and IgA Fc-receptors, hereby obtained, could offer a basis for an understanding of the changes observed during serial mouse-passaging of streptococci (VII). This is further supported by the isolation lately of new types of multicopy and minicircular plasmids from various strains displaying M-protein and opacity factor - but not from their M-negative, opacity factor-negative variants (Golubkov, V.I., personal communication). Thus, available data indicate extrachromosomal regulation of M-protein and Fc-receptors. Conceivably, the changes in production of M-protein and Fc-receptors during passaging of the strains M3, M12 and M46 might be the result of plasmid insertion into the chromosome. Such a hypothesis might also account for the finding (unpublished) that the "P-strains" did not lose Fc-receptor activity or revert to the original M-types during subculturing.

The apparent shift of M-type, while the T-antigen is unaltered, has been designated an "antigenic drift", the originally reported example of which was T12 strains carrying M22 antigen (Maxted & Valkenburg, 1969). T12 strains with other, "new" M-

types have since been identified; several of the new types are "provisional" and monospecific antisera have not been produced (Facklam & Edwards, 1979). Notably, it was found that wild M12 strains invariably lack Fc-receptors, while M22 always seem to have IgG Fc-receptors (Burova et al., 1981). Thus, the "change" from M12 to M22 seems to be accompanied by the acquisition of Fc-receptor activity.

Potter et al. (1974) repeatedly, during epidemics of glomerulonephritis, observed the emergence of strains diverging from "classical" strains by carrying more than one M-antigen. Similarly, Wiley & Wilson (1961) found that type 14 strains, wild or mouse-passaged, often displayed an M-antigen additional to M14, which was named M51. Thus, the streptococcal type may sometimes show instability during epidemics or passaging in mice or human blood.

The majority of group A streptococci carry a set of T-antigens, viz. T-complex. While the same T-antigen(s) may be shared by the strains of different M-types, a given M-type is held to exhibit a defined T-agglutination pattern. However, exceptions to this rule have been noted. For example, Lancefield (1940) described an M3 strain containing T1 antigen in addition to T3, and a "non-typable" T1 strain was given the provisional M-type 68 (Facklam & Edwards, 1979). It is of particular interest in this context that M1 strains may belong to T-types other than T1, in contrast to usual typing experience (see Maxted & Widdowson, 1972). Thus, Potter et al. (1974) found M1 strains with T-agglutination pattern 8/25/Imp.19; the strains were atypical also by probably having an M-antigen additional to M1. Whether M1 streptococci, in particular, regularly have Fc-receptors remains to be elucidated. Several papers have shown that M1 strains may absorb heterologous antibodies from rabbit immune sera or IgG from normal sera (see e.g. Wittner, 1976; Danilova & Borodiuk, 1981). Pellegrino et al. (1972) reported that all human sera (including HLA typing sera) contain antibodies against M1, a finding which might be explained through Fc-receptors. Conceivably, the finding by Hirata & Terasaki (1970) that streptococcal M1 protein inhibited all tested alloantisera against human lymphocytes could also be attributed to an IgG Fc-receptor activity in the preparation.

As stated above, the serially mouse-passaged strains (VII) were not distinguishable from M1 strains. It is therefore possible that the changes observed during mouse-passaging might represent what was earlier described as a "shift of M-type". To the author's knowledge, no previous reports have indicated change to M1, in consequence of mouse-passaging or during epidemics, to be commonplace. However, it appears, interestingly, that most strains used as M1 reference strains have been serially mouse-passaged.

The IgG Fc-receptor of an M1 reference strain (40/58) was isolated by the procedure used for purification of type 15 Fc-receptor (as in VI). It was found that the M1 type antigen and the purified Fc-receptor showed interference with complete fusion in diffusion-in-gel. Furthermore, the M1 antigen was retained on an immunosorbent of human monoclonal IgG coupled to Sepharose (unpublished data). The results indicated that the M1 protein was identical or associated with the Fc-receptor.

In conclusion, there are objections to a contention that the results noted during serial mouse-passaging of some strains (VII) were due to contamination of the strains with M1 streptococci. This possibility was also less probable as the same results were obtained when serial mouse-passages were performed repeatedly with M12. Further studies are required to explain in detail the genetic shift apparently taking place in some streptococci under certain conditions, e.g. mouse-passage or human infection.

3. Increased Susceptibility of Mice to M12 Streptococci after Injection of Purified Fc-Receptor

In order to test the role of Fc-receptor in streptococcal virulence, mice were injected intravenously with partially purified type 15 IgG Fc-receptor and later challenged intraperitoneally with M12/2 or M12/3, both lacking Fc-receptor activity (VII). The Fc-receptor was prepared by affinity chromatography of alkaline extract using the IgG fraction of a normal human serum coupled to Sepharose 4B. All steps were performed in the presence of benzamidine chloride and iodoacetic acid. The preparation, agglutinating Ripley-coated cells at titre 1:20,000, showed contamination by peptidoglycan. Therefore, peptidoglycan, prepared from group A type 15 by formamide extraction (Fuller, 1938) and gel filtration on Sephadex G200 (using the material eluted in the void volume) was included as a control.

It was found that LD of M12/2 or M12/3 was lowered approximately 100-fold in the mice preinjected with purified Fc-receptor, as compared with the animals given saline or purified peptidoglycan (Table 5). These results demonstrated that the streptococcal IgG Fc-receptor preparation, when injected in combination with living Fc-receptor-negative, but M-protein-positive, type 12 streptococci, markedly increased the virulence of this particular bacterial strain. Dose-dependence and effect of injection of chemically pure Fc-binding material in combination with other strains remain to be studied.

Table 5. Influence of partially purified IgG Fc-receptor of type 15 group A streptococci on virulence for mice of strains M12/2 and M12/3. 0.5 ml of the Fc-receptor preparation, peptidoglycan, or saline was injected i.v. 1 h before i.p. injection of ten-fold dilutions of the bacteria

Preparation injected i.v.	Strain injected i.p.	No. mice killed/No. mice injected by		
		10 ⁻²	dilution 10 ⁻³	10 ⁻⁴
Fc-receptor	M12/2	2/2	1/2	1/2
Saline	M12/2	0/2	0/2	0/2
Peptidoglycan	M12/2	0/2	0/2	0/2
Fc-receptor	M12/3	5/5	3/5	3/5
Saline	M12/3	3/5	0/5	0/5

4. Discussion of Possible Role of IgG Fc-Receptors as Virulence Factors

Some authors have proposed that factors other than M-protein and hyaluronic acid may be important for streptococcal virulence. Becker et al. (1973), in particular, found evidence of "additional" virulence factors as the content of M-protein and hyaluronic acid in some variants, selected by blood passage, did not correlate with the virulence for mice. Of special interest is also the finding by Wittner (1976) that serially mouse-passaged streptococci belonging to eight different types were able to absorb opsonic and precipitating antibodies from a heterologous rabbit antiserum. Retrospectively, it seems possible that these variants might have been rich in IgG Fc-receptors.

The present study (VII) showed that the virulent variants selected by repeated mouse-passaging of three group A streptococcal types were devoid of the original M-proteins but had acquired IgG Fc-receptor activity. It appears thus that the Fc-receptors may be involved in determining streptococcal virulence for mice. Results consistent with this theory were also obtained by injecting mice with partially purified type 15 IgG Fc-receptor before challenge with living M12 streptococci. However, the basis for this postulated action is less clear. It is known that streptococcal Fc-receptors have only low affinity for mouse IgG (VIII; Myhre & Kronvall, 1980c).

In conclusion, the data obtained by mouse-passaging and by direct injection of solubilized Fc-receptor indicate a possible role of the IgG Fc-receptors for streptococcal virulence in mice. That the Fc-receptors account for resistance to phagocytosis in humans too was suggested by the high growth indices found in the serially mouse-passaged variants (VII). It is of interest that fresh human isolates often display IgG Fc-receptors (Havlíček, 1978; Burova et al., 1981).

E. STUDIES ON OPSONIZATION OF MOUSE-PASSAGED, VIRULENT STREPTOCOCCI DISPLAYING IgG Fc-RECEPTORS

It is well established that M-positive group A streptococci are opsonized by homologous anti-M antibodies, a property commonly used for definite M-typing (Lancefield, 1962). It emerged in the present study (VII) that the variants obtained by serial mouse-passaging may exhibit loss of the original type-characteristics as judged from indirect bactericidal tests. The present part of the thesis concerns the requirements for opsonization of such highly virulent strains. The variant M12/25, isolated after 25 mouse-passages of M12 as described above was designated 12P; similarly, the variants obtained by 25 passages of M3 and M46 were designated 3P and 46P.

1. Mouse-Protective Effect of Immune IgG F(ab')₂

The LD₁₀₀ for mice of variant 12P, derived from M12, was only 5 CFU. It was demonstrated that rabbit antiserum against 12P and IgG F(ab')₂ thereof, obtained by pepsin digestion, protect mice from challenge with 10,000 and 1,000 lethal doses of 12P, respectively (VIII). Similarly, anti-3P serum and its IgG F(ab')₂ were protective for 12P (Schalén et al., in press). In contrast, no protective effect was obtained with anti-M3 or anti-M12 sera or IgG F(ab')₂ thereof. As 12P and 3P, devoid of original type-antigens, showed IgG Fc-receptors, one explanation of the opsonic effects of IgG F(ab')₂ could be an antibody activity directed against the Fc-receptors. However, it is not known whether such antibodies exist.

2. Induction of *In Vitro* Opsonization by Removing the Fc fragment from IgG of Certain Sera - an Effect Due to Anti-IgG

In analogy to the mouse-protection tests, it was found that IgG F(ab')₂ of anti-3P (Schalén et al., in press) and anti-12P retained the *in vitro* opsonic capacity for 12P, shown by the native sera. Similarly, IgG F(ab')₂ of anti-M3 and -M12, like the native sera, were opsonic for strains M3 and M12, respectively. While anti-M3 and -M12 sera had no effect on strain 12P, the corresponding IgG F(ab')₂, however, were clearly opsonic, though displaying bactericidal indices significantly lower than IgG F(ab')₂ of anti-3P and -12P (VIII; Table 1). Normal rabbit IgG F(ab')₂ was without effect on any of the strains. Hence, removal of the Fc-fragment of IgG caused the antibodies raised against M3 and M12 to opsonize 12P *in vitro*, contrasting to the findings in mouse protection tests. This paradoxical effect could apparently not be due to antibodies directed against type 3 or 12 M-protein, as variant 12P lacked both these proteins. On the other hand, as 12P displayed IgG Fc-receptors it was possible that antibodies with anti-IgG specificity might bind normal IgG linked to the streptococcal surface through Fc-receptors, thereby opsonizing the bacteria. The fact that strain 12P was

opsonized only by the IgG F(ab')₂ but not by the native anti-M12 and anti-M3 sera - might then be explained by binding of the intact anti-IgG antibodies to streptococcal IgG Fc-receptors preventing these from acting as opsonins. This would also presuppose that the anti-IgG antibodies were of the IgG class. This hypothesis is schematically outlined in Fig. 1, paper VIII.

In support of the hypothesis, it was found that absorption of the fragments with red cells sensitized with rabbit IgG abolished the capacity of the F(ab')₂ of anti-M12 to opsonize 12P, while the opsonic capacity for M12 was retained. Furthermore, on passaging of the IgG F(ab')₂ (derived from anti-M3) on IgG-Sepharose, the opsonic capacity for 12P was recovered in the fractions bound and eluted at low pH from the immunosorbent; the homologous strain, M3, was opsonized by the fractions not binding to IgG-Sepharose. The results demonstrated that opsonization *in vitro* of 12P by IgG F(ab')₂ from anti-M3 and anti-M12 was ascribable to the fragments with anti-IgG specificity. In contrast, opsonization of the respective homologous strains was apparently mediated mainly by anti-M IgG F(ab')₂.

In contrast to the effect in bactericidal tests, anti-M3 and anti-M12 antibodies did not induce mouse-protective activity for 12P on removal of the Fc-fragment of IgG. Thus, anti-IgG was without effect in the mouse system. This was not surprising, since the Fc-receptor of 12P, similar to those of other group A strains (Myhre & Kronvall, 1980c), was capable of binding human and rabbit, but not murine, IgG (VIII, Table 3). In addition, the anti-IgG of the isolated IgG F(ab')₂ bound to human IgG, whereas no affinity for mouse, and low for rabbit, IgG was found. Thus, in the protection tests, significant amounts of mouse IgG could not bind to streptococcal Fc-receptors. Furthermore, rabbit IgG F(ab')₂ with anti-IgG activity would obviously not bind mouse IgG molecules to any great degree if attached to the bacterial Fc-receptors.

The present results (VIII) confirmed the previous findings of Perkins & Hahn (1968) and Saito (1969) that the Fc-fragment of IgG is not required for opsonization of streptococci in bactericidal tests or in the mouse system; notably, these investigators used repeatedly mouse-passaged strains. In contrast, opsonization of other species, e.g. *E.coli* by IgG is allegedly dependent on the Fc-part, eliciting complement activation through the classical pathway (Spiegelberg et al., 1963). Furthermore, an "aspecific", complement-dependent opsonization, mainly of non-encapsulated bacteria, by Fc-fragments or monoclonal IgG was reported (Stinson & van Oss, 1971). It is known that opsonization also of virulent streptococci requires the participation of heat-labile serum factors (Stollerman et al., 1963). However, it was found that the second component of complement, C2, is not contributory (Stollerman et al., 1967). As aggregated rabbit IgG F(ab')₂ was demonstrated to activate the alternative complement pathway (Reid, 1971) it seems possible that virulent streptococci, coated with IgG Fab or F(ab')₂ - like avirulent streptococci

(Bisno, 1979) - may be opsonized through activation of the alternative pathway.

Recently, purified IgG Fc-receptor from type 15 group A streptococci was shown to interfere with complement-dependent lysis of sheep red blood cells (SRBC) sensitized with rabbit anti-SRBC of the IgG (but not IgM) class, indicating that the binding of the Fc-receptor to IgG could block complement fixation (Burova et al., in press). Conceivably, the capacity of the streptococcal factor to inhibit classical pathway activation may explain why the Fc-fragment seems not to contribute to the opsonization of certain streptococci, in particular.

Opsonization of virulent streptococci, displaying Fc-receptors, by anti-IgG antibodies is a new observation. This mechanism was the plausible explanation for the finding that variant 12P was opsonized *in vitro* by IgG F(ab')₂ of certain rabbit sera - but not by the native sera. Interestingly, anti-IgG (of the IgM class) was recently demonstrated to opsonize *Trypanosoma lewisi* (Clarkson & Mellow, 1981) which also may exhibit IgG Fc-receptors (Ferreira De Miranda-Santos & Campos-Neto, 1981). A role of the streptococcal Fc-receptors for triggering of anti-IgG was reported recently (Lebrun, 1981). It remains to be studied whether also intact anti-IgG antibodies may act as opsonins for streptococci displaying Fc-receptor activity.

Summary

Streptococcal immunoglobulin receptors were originally demonstrated in 1973. Group A strains, belonging to several types, and the majority of group C and G strains have been shown to bind the Fc-part of human and rabbit IgG. Radiolabelled myeloma proteins of all four human IgG subclasses were bound to group A, C and G Fc-receptors. Subsequent studies with sera and IgG from various animals, however, showed affinity differences between group A and group C and G strains as well as between various species of group C and G streptococci. It is also known that a minority of group A streptococci are capable of binding IgA through determinants not involving the antigen binding regions. The immunoglobulin receptors are sensitive to trypsin treatment and were shown by electron microscopy to be present at the filamentous surface projections of streptococci.

On absorption of sera with streptococci by procedures conventionally used for typing purposes, binding of IgG to Fc-receptors may non-specifically deplete rabbit immune sera of the bulk of IgG; thus, difficulties in streptococcal typing and erroneous interpretations of experiments allegedly showing cross-reactions between streptococci and mammalian tissue may arise. While in most of the previous work by others the immunoglobulin receptors were studied on intact bacteria, the solubilization of the receptors by some methods, viz. acid or alkaline hydrolysis, autoclaving or phage-associated lysis, was also achieved. The solubilized receptors were detected by agglutination of sheep red blood cells sensitized with rabbit antibodies against such cells. Furthermore, a partial purification of an IgG-binding substance of group C streptococci was accomplished.

The present thesis mainly concerns the immunoglobulin receptors of group A streptococci and their reactions with human immunoglobulins. The class and intraclass specificities of the solubilized receptors from group A strains representing various types were investigated by agglutination and gel-precipitation methods. In type 15, particularly studied, the solubilization conditions were found to influence on the specificity of the receptor for the Fc-fragment of human IgG3. Moreover, the gel-precipitating properties of this receptor were found to vary

depending on the composition of the gel. The part of the IgA molecule reacting with the IgA-receptor was determined in binding experiments with intact bacteria. The relations of some immunoglobulin receptors to other streptococcal components were studied. An IgG Fc-binding substance was isolated, using a new method, and partially characterized. In addition, the possible influence of the IgG Fc-receptors on streptococcal virulence and on the opsonization of streptococci was elucidated.

A. Demonstration of Solubilized Streptococcal IgG and IgA Fc-Receptors

The presence of solubilized receptors for IgG Fc in the acid extracts of various types of group A and of one strain each of group C and G streptococci was demonstrated by agglutination of human anti-Rh coated red blood cells. As expected IgG Fc, but not Fab, was inhibitory in such systems.

Furthermore, the IgG Fc-receptor of a group A, type 15 strain, solubilized by acid or alkaline hydrolysis, precipitated polyclonal human IgG in gel. Electrophoretic separation of type 15 alkaline extract showed the receptor to migrate slowly at pH 8.6. The precipitate formed between this substance and IgG was more anodally positioned on addition of IgG Fc to the extract before electrophoresis. A precipitating IgA receptor in the alkaline extract of a group A, type 4 strain was similarly identified by addition of IgA to this extract. However, the type 4 extract also contained an IgG Fc-receptor, though with low precipitating capacity under these conditions. The receptors for IgA and IgG, respectively, in type 4 were shown to be separate substances. By using IgA Fc and Fab as inhibitors for the binding of IgA to intact bacteria it was shown that the IgA-receptor in type 4 streptococci interacts with the Fc-fragment. The interaction between type 4 streptococci and IgA did not induce fragmentation of the IgA molecule, indicating absence of IgA-cleaving enzyme in this strain (as in other group A streptococci).

B. Differential Reactivity of Streptococcal Fc-Receptors for Human IgG. Influence of Solubilization Conditions on the Fine Specificity and Precipitating Properties of One Receptor

The IgG Fc-receptors from various streptococci, solubilized without presence of protease inhibitors, at various titres agglutinated human red blood cells sensitized with IgG1 and IgG3 anti-Rh. The specificity for IgG was further characterized by using purified myeloma proteins as inhibitors in the tests. A minimum of four different IgG affinity types of Fc-receptors were distinguished in group A, C and G streptococci. The IgG Fc-receptor of type 15 exhibited variable precipitation frequencies with normal sera from different populations. The molecules in the precipitate belonged to IgG3 and at least one of the other IgG subclasses. IgG-binding material purified from this extract showed a preferential affinity for the genetic marker G3m(5) as compared with G3m(-5). Thus, the affinity for

human IgG Fc was iso-allotypically restricted.

To create precipitate in plain agarose gel between human serum or myeloma proteins and the IgG Fc-receptor of type 15, solubilized without protease inhibitors, contribution of IgG molecules reacting with the Fab-parts was required. Furthermore, the tendency to precipitate formation between this Fc-receptor and human serum was augmented by addition of 10 % dextran T10 to the gel, increasing the proportion of precipitating normal Swedish sera from 82 to 100 %.

The Fc-receptor activity of various solutions often decayed during solubilization or storage, which was ascribed to presence of streptococcal protease(s). Thus, by addition of protease inhibitors during solubilization degradation of Fc-receptors was prevented. In addition, the Fc-receptor of type 15 streptococci, solubilized in the presence, as compared to absence, of protease inhibitors displayed a higher precipitating capacity, enabling precipitation in plain agarose gel of 100 % of normal sera. The receptor thus solubilized was unrestricted in the affinity for the Fc-fragment of all subclasses of human IgG. Moreover, as this receptor precipitated IgG Fc-fragments, it was obviously not dependent on binding through the Fab-part for precipitation.

C. Further Characterization of Streptococcal Immunoglobulin Receptors. Relation to Other Streptococcal Components and to Staphylococcal Protein A

The respective Ig-receptors of type 4 and 15 group A streptococci were shown to be separate from various cell wall components, e.g. peptidoglycan, group-specific carbohydrate, lipoteichoic acid and M-protein. The relation between the immunoglobulin receptors and the M-associated proteins, however, is still unresolved.

Type 15 IgG Fc-receptor was purified from alkaline extract by ion-exchange chromatography on DEAE-cellulose followed by bioaffinity chromatography on IgG-Sepharose; each step was performed in the presence of protease inhibitors. Despite a MW of 29,500, determined by SDS-polyacrylamide gel electrophoresis, the purified IgG-binding substance, a polypeptide, displayed a hydrodynamic volume close to that of IgA, thus suggesting an elongated shape or a tendency to polymerization. Amino acid analysis revealed a relatively high content of glutamic acid and alanine and low content of tyrosine, proline, and phenyl-alanine. The composition differs from those of some M-proteins investigated and of staphylococcal protein A.

D. Studies on the Importance of IgG Fc-Receptors in Streptococcal Virulence

Group A streptococci, type M12 were serially passaged 25 times in mice. On the fourth passage the M12 protein was found to disappear. The virulent variants selected by the subsequent passages displayed IgG Fc-receptors, in contrast to the original strain. Similar changes were observed on serial mouse-passaging of M3 and M46, group A streptococci. Immunochemical and bactericidal tests showed all the variants selected by serial mouse-passage to react with some anti-M1 sera, suggesting that contamination with M1 might have occurred. However, the T12 antigen was retained during the passages of M12, which did not support such a concept. The interpretation of these changes and their relation to variations in virulence is discussed.

In preliminary experiments, mice were injected i.v. with soluble Fc-receptor of type 15 streptococci and subsequently challenged i.p. with M12 streptococci (lacking Fc-receptors). As a result the LD₁₀₀ of M12 was reduced a 100-fold which suggested that the IgG Fc-receptors may be virulence factors in group A streptococci.

E. Studies on Opsonization of Mouse-Passaged, Virulent Streptococci Displaying IgG Fc-Receptors

The virulent variant 12P, derived from M12 by mouse-passaging, was opsonized *in vitro* and *in vivo* by rabbit antiserum against 12P, as well as IgG F(ab')₂, indicating that antibodies directed against the Fc-receptor might be responsible. However, it remains to be seen whether streptococcal Fc-receptors are antigenic. Anti-M12 or anti-M3 sera or IgG F(ab')₂ thereof did not protect mice on challenge with 12P.

In contrast, in bactericidal tests, anti-M3 and anti-M12 were not opsonic for 12P while the corresponding IgG F(ab')₂ clearly were so. By separating the fragments by bioaffinity chromatography this seemingly paradoxical difference was explained as being due to molecules with anti-IgG specificity. Conceivably, the opsonization of streptococci by anti-IgG - not previously described - occurs through binding to human normal IgG, supplied by the donor blood used in the test and bound to the streptococcal surface through Fc-receptors.

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Appendix

BRIEF REPORT

LANCIEFIELD EXTRACT OF GROUP A STREPTOCOCCI TYPE 15 ACTS LIKE AN ANTI-HUMAN IgG WITH RESTRICTED SPECIFICITY

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Schalén, C., Christensen P. & Grubb, R. Lancefield extract of group A streptococci type 15 acts like an anti-human IgG with restricted specificity. Acta path. microbiol. scand. Sect. C, 86: 41-43, 1978.

Lancefield extract of streptococci group A type 15 precipitates certain human sera and agglutinates red cells coated with some IgG anti-Rh antibodies. The frequency of precipitation varies between populations. The serum component in the precipitate is IgG. The results indicate that an iso-allotypic marker is detected by the extract.

Key words: Lancefield extract; streptococci; anti-human IgG; iso-allotypes; Gm markers; human Ig.

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In 1959, Wood & Schramm (12) described a capillary precipitation reaction between Lancefield-extract of group A, type 15 streptococci and all of the 137 human normal sera tested. In some respects the streptococcal factor responsible for the precipitation resembled the M protein, while the serum factor participating was not characterized.

Many streptococci, including group A, type 15, have receptors for human IgG (2, 4, 8). Extracts *ad modum* Lancefield are known to contain these receptors (3). It was thought worth while to find out whether this Lancefield extract had in fact properties of an anti-human gammaglobulin.

Materials and Methods

Streptococcal extract. Group A streptococci, type 15 (EF 1949), were cultured on 4 × 5 l Todd Hewitt broth, containing 0.5% glucose, at 37°C for 6-8 hours using magnetic stirrers; each 5 l was inoculated with 0.3 l over night culture of type 15. pH was adjusted to 7.0 every 30-60 min with 2 N NaOH during growth. The bacteria were harvested in a continuous action rotor in a Hi-spin 21 centrifuge (MSE, Sussex, England) at 9,000 rev/min. Forty ml wet volume of bacteria washed in 0.9% saline was suspended in 100 ml saline and the pH lowered to 2.0 by HCl. The suspension was heated in a boiling waterbath for 10 min, after which the pH was adjusted to 7.0 with NaOH, the bacteria were centrifuged and the

supernatant was removed and kept at 4°-8°C. The following day a cloudy precipitate was removed by centrifugation at 3,000 g. The final volume of this extract, used in all experiments, was 150 ml and the protein content, determined as described (10), 2 mg/ml. Many of the experiments were repeated several times with minor lots of Lancefield extracts of cultures obtained on separate occasions.

Human sera. Some of the sera had been stored for more than 10 years at -20°C. All sera were heated at 56°C for 30 min before use. The IgG, IgA and IgM content of some sera was determined by the electroimmunoassay (9).

Separation procedures. Human commercial IgG and antisera. Sera were separated by means of a Sepharose 4B-protein A column from Pharmacia (7). A column of human commercial IgG-Sepharose 4B was used, as earlier described (1). All fractions obtained from the columns were concentrated to the volumes of the specimens applied.

Commercial human IgG was purchased from Kabi, Stockholm, Sweden, and rabbit antisera to IgA, IgG and IgM from Dakopatts, Copenhagen, Denmark. The anti-IgG3 was a gift from Dr. A. Grubb, Malmö.

Tube and gel precipitation. Gel precipitation tests were performed as earlier described (11). The precipitation lines between M 15 extract and human sera were visible after 1-2 days.

In tube precipitation experiments, 1 ml of serum was mixed with 1 ml of M 15 extract and 4 ml of 0.9 per cent

saline added. After 2 hours at 37°C and 3 days at 4°–8°C the precipitate was spun down at 8,000 g for 1 hour and washed once. 0.2 ml PBS was afterwards added. The precipitate was dissolved by incubation at 37°C with 20 µl trypsin (Sigma, St. Louis, M.O., USA) 0.6 mg/ml (the M 15 precipitating factor is trypsin sensitive; see Wood & Schramm (12)). Trypsinization was stopped by 20 µl soy bean inhibitor (Sigma, St. Louis, M.O., USA) 5 mg/ml. Controls included 1) only serum or only M 15 extract, trypsinized as indicated above, and 2) only trypsin and inhibitor.

Agglutination and agglutination inhibition tests with cells coated with incomplete anti-Rh were performed as in Gm-typing procedures (5) using plastic trays as described (6). The anti-Rh's used included the well known Ripley serum, which was a gift from Dr. Marion Waller, Richmond.

Results

Gel Precipitation with Human Sera from Various Populations

The Lancefield extract precipitated human sera as follows:

East Greenlanders	3 out of 33	or	9 per cent
Laplanders	27 out of 36	or	75 per cent
South Koreans	25 out of 25	or	100 per cent
Swedes	32 out of 39	or	82 per cent.

Precipitation was usually obtained also with serum diluted 1:4. A few sera gave 2 lines. All sera classified as precipitated gave a line which fused with that of a control serum.

Characterization of the Precipitated Component of Human Serum

All of 21 sera chosen for testing because they contained <7 mg IgG per ml failed to give a line in gel precipitation tests. Four of 8 sera containing >7 mg IgG but less than 0.7 mg IgM per ml were precipitated, however, and the same was true for 2 out of 5 with a level of IgA below 0.7 mg/ml.

In diffusion in gel experiments the precipitation line formed between anti-IgG3 and serum fused with the line obtained between the same serum and the type 15 Lancefield extract. Our anti-total-IgG gave broad, indistinct precipitates making the result of the Ouchterlony comparative analysis less clearcut.

Precipitates in tubes were obtained from 8 »positive« human sera. The precipitates were dissolved as described in »Methods«. All 8 preparations were precipitated by anti-human IgG and anti-human IgG3, but not by anti-human IgA or anti-IgM in gel precipitation.

The column containing staphylococcal protein A coupled to Sepharose used is known to retain IgG of subclasses 1, 2 and 4 and to let IgG3 run through. The fraction eluted and the fraction retained were both precipitated by the M 15 extract. The precipitates showed Ouchterlony identity reactions with each other and with the line obtained with unseparated serum.

Commercial human IgG KABI was precipitated by the extract but only at Ig concentrations of at least 25 mg per ml. This IgG is derived mainly from retroplacental blood

and this prompted us to investigate the precipitating capacity of sera from pregnant women. It was found that 9 out of 45 sera from pregnant women (32nd–40th week of pregnancy) precipitated. This figure was significantly lower than that found in non-pregnant Swedes (see above). Further observations related to this finding will be published separately. Unless otherwise stated, no sera from pregnant women were included in the material forming the basis of this communication.

Agglutination of Red Cells Coated with Incomplete Anti-Rh and Results of Agglutination-Inhibition Tests

Lancefield extracts diluted several thousand times agglutinated group ORh + red cells coated with anti-Rh Ripley diluted 1:10 at coating. Uncoated red cells were not agglutinated. Trypsin-treated extracts did not agglutinate the coated red cells, neither did extracts which had passed an IgG coated Sepharose column. The agglutination by extracts diluted to contain 8–16 agglutinating doses was inhibited by KABI gammaglobulin in a concentration of less than 0.01 per cent.

In preliminary tests we assessed the ability of the extract to agglutinate samples of erythrocytes coated by 12 other incomplete anti-Rh which were of approximately the same titer as judged by Coombs' test using rabbit antihuman-gammaglobulin. Agglutination was obtained with 7 of the coated cell samples. As expected, the titer was not as high as with the Ripley coated cells. It was noteworthy that some high-titred anti-Rh did not coat for agglutination by the Lancefield extract. Individual sera were tested for their capacity to inhibit agglutination of red cells coated with anti-Rh Ripley as well as with an anti-Rh HUN found useful for typing IgG3 Gm markers. Most sera were inhibitory at a dilution of at least 1:25. A small percentage of sera were markedly less inhibitory and quite a few sera were in an intermediate range. Sera from pregnant women were inhibitory to the same extent as other categories of sera. Nine pairs of mother-newborn sera were examined and the two sera of each pair were equally inhibitory.

Discussion

The precipitates observed might be considered to consist of M-protein type 15 and homologous antibodies. Such an interpretation is in accordance with the observation that the reacting component of serum is IgG and could, perhaps, also account for the varying incidence of precipitation in the populations studied. This interpretation is less likely for the simple reason that type 15 infections are uncommon and, secondly, also because it is known that human antistreptococcal antibodies rarely precipitate extract of streptococcal cells (12). Such interpretation fails to account for the observed agglutination of red cells coated with IgG anti-Rh-antibodies. An alternate explanation is that this Lancefield extract interacts with human Ig irrespective of combining sites for streptococci or streptococcal products. In our opinion, the data presented make it difficult to avoid this conclusion. We thus ascribe the precipitation and the agglutination of anti-Rh-coated red cells to the content of Ig Fc receptors in the streptococcal extract.

Much evidence, viz. the existence of precipitating and non-precipitating sera, the ethnic group differences, the differential action of the extract on a number of anti-Rh coats, the variation in the capacity of individual sera to inhibit the haemagglutination observed, suggests that the streptococcal extract interacts with some IgG molecules but not with others. As for the nature of this restriction, an important clue is that reacting IgG molecules were shown to be present not only in the IgG3 subclass but also in at least one of the other subclasses. All known IgG allotypic markers are confined to one subclass (see Grubb (5)). This observation alone indicates that the restriction is due to a marker with the character of an iso-allotype. The results of the haemagglutination inhibition tests also lend support to this interpretation.

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INTERACTION OF THE Fc PART OF IgG WITH LANCEFIELD EXTRACTS OF HEMOLYTIC STREPTOCOCCI

Strain Specificity and Activity

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Christensen, P., Burova, L. A., Grubb, A., Grubb, R., Samuelsson, G., Schälén, C. & Svensson, M.-L. Interaction of the Fc part of IgG with Lancefield extracts of hemolytic streptococci. Strain specificity and activity. Acta path. microbiol. scand. Sect. C, 87: 73-77, 1979.

Lancefield extracts of 19 types of group A streptococci as well as one group C and one group G strain were examined for agglutination of human red cells coated with various anti-Rh antibodies. Fourteen extracts agglutinated one or more of the coated cell samples, while five did not. The agglutination was inhibited by Fc but not by Fab fragments of human IgG. After mouse passages, three of the non-agglutinating strains acquired agglutinating capacity. At least three different reactivities were distinguished by the action of the extracts on IgG1 and IgG3 coated cells, respectively. Two of the streptococcal extracts, agglutinating the same anti-Rh coated cells, could be further differentiated in hemagglutination inhibition (HAI) experiments using purified IgG3 myeloma proteins. Five selected agglutinating systems were inhibited by purified myeloma proteins of the IgG1, IgG2 and IgG4 subclasses. IgG3 proteins inhibited only two of the five HAI systems.

Key words: Streptococci; Lancefield extract; iso-allotypes; Gm markers; human Ig; Fc fragment.

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The capacity of Lancefield extracts of group A streptococci to agglutinate sheep red cells coated with rabbit anti-sheep red cell antibodies (SRBC) was demonstrated in 1974 (4). This reactivity is widespread among group A streptococci (8). Recently, Lancefield extract of group A type 15 streptococci was found capable to agglutinate Rh-positive cells coated with »incomplete« anti-Rh antibodies and to form precipitate in gel with human sera. The reactivity of this extract was restricted as determined with sera from various populations and its capacity to agglutinate red cells coated with various anti-Rhs differed (15). In the

present paper, extracts from different streptococcal groups and types were investigated for restriction in affinity for human Ig.

MATERIALS AND METHODS

Streptococcal Strains

The following reference strains of group A streptococci were used: M3 (BG 930/24), M5 (100065), M6 (8302), M8 (8324), M9 (100067), M11 (100068), M12 (R53), M15 (100070), M17 (8304), M22 (EF 1950), M27 (EF 1913), M46 (8230), M55 (100189), M56 (100191) and M57 (100190). Furthermore, the following *Griffith* strains were used: T13 (Glover) and T44 (Henson glossy). 81C and 113G, belonging to group C

and G, respectively, have previously been used (5). Group A, T type 28, opacity factor positive (OF+) streptococci were isolated during an epidemic outbreak of streptococcal sore throat in a day nursery. T-typing and test for OF were performed as described (1, 11); 47 of 90 children were infected with the strain.

Mouse Passage of Streptococcal Strains

The strains M3 (BG 930/24), M12 (R53) and M46 (8230) were subjected to mouse passages over several years. Each passage was performed by intraperitoneal injection of mouse spleen suspension taken from dead mice within 24 hours.

Streptococcal Extracts

Group A streptococci, types M15, T28, T44 and M55, were cultured in 20 litres and the other strains in 0.3 litres of Todd-Hewitt broth; cultivation and extraction *ad modum* Lancefield were performed as described elsewhere (15). Several extracts of each strain were prepared and used in repeated experiments.

Human Sera

Sera were obtained from the staff at the above-mentioned day nursery and from the parents of the children. The sera were heated at 56°C for 30 min before use.

M-Components

The M-components were purified from plasma samples from patients with multiple myeloma. A combination of ammonium sulphate precipitation, ion-exchange and gel chromatography and preparative agarose gel electrophoresis (10) was used to isolate the M-components. Their purity was shown by analytical agarose gel electrophoresis (10), immunoelectrophoresis (7) and SDS-polyacrylamide electrophoresis (16). Contaminating proteins were below 2% in the preparations. The M-components were dialysed against distilled water, lyophilized and stored at room temperature. Stock solutions containing 2 mg protein/ml 0.9% NaCl, determined as described (13), were prepared for the experiments.

Determination of the Subclass of the IgG M-Components

The chemical typing procedure of Frangione *et al.* (6) was used.

Fab- and Fc-Fragments of Polyclonal IgG

Polyclonal IgG was prepared from a pool of plasma from several hundred registered blood donors by gel chromatography on DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated in 0.05 mol/l Tris-buffer, pH 7.4. Appropriate fractions were pooled and further purified by gel chromatography on Sephadex G-200 (Pharmacia Fine Chemicals). Fab and Fc fragments of the polyclonal IgG were prepared by mercury-papain digestion, essentially as described by Hsiao & Putnam (9) except that the digestion was terminated after 30 min by addition of recrystallized iodoacetic acid. The fragments were isolated from

undigested IgG by gel chromatography on Ultrogel AcA54 (LKB, Stockholm, Sweden) and the Fab and Fc fragments separated by preparative agarose gel electrophoresis (10). The preparations of the fragments were desalted and lyophilized.

Agglutination and Agglutination Inhibition Tests

These tests were performed essentially as described previously (15). The anti-Rh Ripley (Ri) was a gift from Dr. Marion Waller, Richmond, USA; this anti-Rh is exceptional as it is complement-fixing. Anti-Rh KM and 317 are useful for detection of the genetic markers G1m(1) and G1m(4), respectively. The anti-Rh HUN detects IgG3 markers which are G3m(5) and is as high-titred an anti-Rh as Ri, on analysis with conventional techniques. For coating, the anti-Rhs were diluted as indicated in Table 1.

Demonstration of Opacity Factor (OF) and Anti-OF Antibodies

OF from group A streptococci type T28 was demonstrated as described by Maxted *et al.* (14). The titre of OF-antibodies was expressed as the highest dilution of serum inhibiting the opacity reaction.

RESULTS

Agglutination of Red Cells Coated with Incomplete Anti-Rh by Lancefield Extracts

The hemagglutination titres of the extracts for red cells coated with four samples of high-titred anti-Rh antibodies are given in Table 1. Of the 19 extracts, 14 agglutinated one or more of the coated cell samples. Types M11 and M17 streptococci are excluded, as Lancefield extracts from these two strains weakly agglutinated uncoated red cells. It is apparent that the titres for cells coated with Ri are much higher than for cells with other anti Rhs. Seven of the extracts, including those of the group C and G strains, still agglutinated the Ri-coated cells on dilution more than 1000 times. All extracts with a »Ri-titre« of 640 or more agglutinated the cells coated with at least two other anti-Rhs. The results with the IgG1 coats KM and 317 were very similar. It was notable that the M56 extract was active for the IgG1 coats but was inactive for the IgG3 coat (HUN). This was in contrast to M8, M15, group C and group G, which were active for both, and T44, which was active for IgG3 alone.

Acquisition of Ig Binding Activity by Mouse Passage of Strains

The extracts of the three group A streptococcal strains M3, M12 and M46 marked with »C« in Table 1 did not agglutinate any of the coated cell preparations. After repeated mouse passage of the

TABLE 1. *Agglutination of O Rh+ Red Cells Coated with Human IgG Anti-Rh by Lancefield Extracts of Streptococci^a*

Extract of streptococcal Group Strain	Agglutination titre of extracts for red cells coated by anti-Rh			
	Ri (1:15) ^b	KM (1:5) ^b	317 (1:5) ^b	HUN (1:10) ^b
A	M3 ^c	<10	0	0
	M4	1280	4	0
	M5	40	0	0
	M6	<10	0	0
	M8	20000	8	8
	M9	20	0	0
	M12 ^c	<10	0	0
	T 13	20	0	0
	M15	20000	16	16
	M22	40	0	0
	M27	<10	0	0
	T 28	640	8	0
	T 44	20	0	4
	M46 ^c	<10	0	0
	M55	20000	16	4
	M56	2560	16	0
	M57	<10	0	0
C	81C	2560	8	16
G	113G	2560	32	64

^a The extracts of M11 and M17 are excluded from the table as agglutination of uncoated cells occurred.

^b Indicates the dilution of the anti-Rh at coating

^c Before being subjected to mouse passages. See text.

strains, the extracts agglutinated Ri (diluted 1:15 at coating) in a dilution of 1:180–360 and HUN (1:10) in a dilution of 1:16. KM (1:5) or 317 (1:5) coated cells were not agglutinated.

Myeloma Proteins as Inhibitors of Hemagglutination Systems

As mentioned above, 14 of the 19 samples agglutinated one or more of the four coated cell

TABLE 2. *Hemagglutination Inhibition by Purified Myeloma Proteins*

HAI systems	Number of myeloma proteins inhibitory ^a					
	IgG1	IgG2	IgG3	IgG4	IgD	IgM
M15/KM	5	2	0	3	0	0
M15/HUN	5	2	4	3	0	0
T28/Ri	5	2	0	2	0	0
T44/Ri	5	2	0	3	0	0
113G/KM	5	2	4	3	0	0
Total number of myeloma proteins tested	5	2	4	3	1	3
Total number of HAI systems inhibited	5/5	5/5	2/5	variable	0/5	0/5

^a Inhibitory at a concentration of 0.1–0.01 mg/ml. Myeloma proteins not inhibitory at 2 mg/ml were registered as non-inhibitory.

samples. In all, the results recorded in Table 1 make 36 hemagglutination systems available for inhibition studies. The 18 purified myeloma proteins at our disposition have so far been tested for their capacity as inhibitors in 5 systems, representing different streptococcal agglutinating activities: M15/KM (streptococcal extract/anti-Rh coat), M15/HUN, T28/Ri, T44/Ri and 113G/KM (Table 2). The five IgG1 and the two IgG2 myeloma proteins were inhibitory at 0.1–0.01 mg/ml in all five HAI systems. The IgG3 myeloma proteins were inhibitory only in two, namely M15/HUN and 113G/KM. The IgG4 myeloma proteins were inhibitory in all systems, with the exception of one myeloma protein which was less inhibitory in the T28/Ri system. None of the HAI systems was inhibited by the IgD or the three IgM myeloma proteins (2 mg/ml).

Fab- and Fc-Fragments of Human Polyclonal Ig as Inhibitors

Fc-fragments were inhibitory at 0.01–0.001 mg/ml in all of the five HAI systems tested. No inhibition was obtained by Fab fragments at 0.5 mg/ml.

Lack of Correlation between Anti-OF Antibody Titre of Sera and Inhibitory Titre of Sera in HAI System T28/Ri

Sixty sera sampled from parents and staff engaged in a type T28, SOR + epidemic outbreak in a day nursery were investigated for inhibitory capacity in the HAI system T28/Ri. The anti-OF T28 antibody titres ranged from 1:1 to 1:640 in 21 sera, while 39 did not contain such antibodies. Absence or presence of anti-OF T28 antibodies did not correlate to the inhibitory capacity of the sera, neither did the titres of OF antibodies.

DISCUSSION

It is apparent from Table 1 that *Lancefield* extracts from streptococcal strains vary markedly in their capacity to agglutinate red cells coated with anti-Rh. The titres of the extracts for the »Ri-coated« cells were considerably higher than for the cells coated with the other anti-Rhs. It should be noted that the Ri coat is exceptional among anti-Rhs, since it detects rheumatoid factors much more effectively than do other coats. The reasons for this behaviour of Ri are not well understood. Extracts from strains M8, M15 and M55 could be diluted more than 20,000 times and still agglutinate the red cells coated with Ri, whereas the undiluted extracts of M3, M6, M12, M27 and M46 were inactive. All strains except T28, 81C and 113G were reference

strains, and have thus been recultivated in the laboratory for several years.

The extracts of strains M3, M12 and M46 agglutinated both Ri-coated and HUN-coated cells after mouse passage, in contrast to the extracts obtained before the passages, which were inactive. These strains were M-negative after the prolonged passages yet highly virulent for mice, as compared with the original strains (2). Studies are in progress to elucidate whether the agglutinating capacity can also be increased in this way in strains showing low activity, or induced in other »negative« strains. *Havlicek* (8) found that all of 38 fresh isolates from an epidemic outbreak agglutinated SRBC, while only 38 of 175 collection strains did so. This might indicate that the agglutinating capacity could be lost during storage of the strains. However, it remains to be clarified whether some strains of certain types might lack capacity to agglutinate anti-Rh coated cells also when freshly isolated from clinical sources or after mouse passages. *Havlicek* (8) found no activity for SRBC among 28 type 12 group A streptococci, 13 of which were collection strains and 15 freeze-dried cultures. On the other hand, our results might indicate that the capacity to agglutinate sensitized red blood cells is inducible in type 12 and lack of this characteristic thus may not be a constant property of this particular type.

Only IgG proteins were inhibitory in the five HAI systems tested. Neither the IgD nor three IgM myeloma proteins were inhibitory in any of the systems; IgA proteins have so far not been studied. As inhibition was obtained by Fc fragments of IgG but not by Fab fragments, it is reasonable to assume that the »anti-Ig activity« in the streptococcal extracts is identical with the IgG Fc receptor of streptococci (3). Furthermore, *Havlicek* (8) found that the Fc part of rabbit IgG inhibited streptococcal agglutination of SRBC.

The *Lancefield* extracts of M15, T44 and M56 streptococci gave different agglutination patterns with red cells coated with KM and HUN anti-Rhs, useful for detection of IgG1 and IgG3 genetic markers, respectively. To further elucidate the restrictions in reactivity for human immunoglobulins expressed for the different extracts, the inhibitory effect of myeloma proteins on the hemagglutination was studied. The reactivity of the extracts in HAI experiments varied from one streptococcal type to another. We therefore considered the possibility that inhibition by a myeloma protein in one system but not another could be due to antistreptococcal type-specific antibodies. Test of a number of human sera with and without type-specific antibodies for the T28 strain in the T28/Ri HAI system showed no correlation between inhibition and presence of

type-specific antibodies. However, it is necessary to further elucidate the possible influence of antibodies directed against other streptococcal components, before the outcome of HAI studies with normal sera can be attributed to inhibition only by the Fc parts.

The M15 and 113G extracts were similar in the sense that they agglutinated both KM and HUN sensitized cells. However, in HAI systems involving the KM coat and IgG3 myeloma proteins, these extracts behaved differently (Table 2). Including the finding that some extracts were non-reactive with anti-Rh coated cells, five different affinities for human IgGs could be registered, exemplified by: M3 (non-reactive), M4 (reactive with IgG1 coats only), T44 (reactive with the IgG3 coat only) and M15 and 113G (reactive with coats of both these subclasses but differing in HAI systems). The restricted activity of the M15 extract was further illustrated by the finding that the four IgG3 myeloma proteins inhibited the agglutination of the IgG3 coated cells but not of the IgG1 coated cells (Table 2).

The five IgG1 proteins belonging to G1m(1) or G1m(4) all inhibited the five HAI systems investigated, indicating that the inhibition was caused by a marker that is isotype for the IgG1 subclass. The observations of *Kronvall* (12) indicated differences between two IgG3 myeloma sera in affinity for a group A streptococcal strain. All the IgG3 myeloma proteins used in the present study were Gm(5), which renders it less likely that these proteins should differ with respect to genetic markers in the Fc part. It was also found that all four IgG3 myeloma proteins varied in the same way in the different HAI systems. That differences within one IgG subclass could be detected in HAI systems involving streptococcal extracts was elucidated by the differing behaviour of one of the three IgG4 myeloma proteins in the T28/Ri system. The similarity in the inhibition patterns of the IgG1 myeloma proteins, combined with the results obtained with the IgG4 myeloma proteins, may indicate that some of the extracts have affinity for iso-allotypic markers as our earlier experiments have suggested (15).

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DEMONSTRATION OF THE NON-IDENTITY BETWEEN THE Fc RECEPTOR FOR HUMAN IgG FROM GROUP A STREPTOCOCCI TYPE 15 AND M PROTEIN, PEPTIDOGLYCAN AND THE GROUP SPECIFIC CARBOHYDRATE

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Christensen, P., Grubb, A., Grubb, R., Samuelsson, G., Schalén, C. & Svensson, M.-L. Demonstration of the non-identity between the Fc receptor for human IgG from group A streptococci type 15 and M protein, peptidoglycan and the group specific carbohydrate. Acta path. microbiol. scand. Sect. C, 87: 257-261, 1979.

After electrophoresis of an alkaline extract of type 15 group A streptococci, three main precipitation lines were obtained in diffusion experiments against commercial human polyclonal IgG (lines 1, 2 and 3). Nineteen of 23 sera (83%) from apparently healthy human individuals gave line 3, while 6 of them (26%) gave line 1. The sera giving line 1 did also give line 3. Line 2 was obtained with 2 sera only, also giving lines 1 and 3. Line 3 was caused by a streptococcal Fc-receptor for human IgG, since the line could be displaced by addition of Fc-fragments, but not Fab-fragments of pooled human IgG. Line 1 was shown to be different from line 3, since (1) line 1 was suppressed in contrast to line 3 on absorption of a human serum or commercial polyclonal human IgG with *S. aureus*; and (2), line 1 was suppressed by Fab-fragments but not Fc-fragments of polyclonal human IgG. Line 2 could be inhibited by addition of peptidoglycan to commercial polyclonal human IgG or a human serum investigated. Another line, 4, obtained in diffusion experiments involving electrophoretically separated alkaline extract of type 15 group A streptococci was type-specific as shown by rabbit antisera to streptococci type M1, M8, M15, and T44, and disappeared on trypsinization of the extract. The component responsible for line 4 in the streptococcal extract, judged to be type-specific M protein, had a mobility different from the component responsible for line 3 in electrophoresis.

Key words: Streptococci; M protein; peptidoglycan; group A carbohydrate; human IgG.

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Hot hydrochloric acid extract ad modum Lancefield of type 15 group A streptococci is capable of precipitating IgG in a majority of human sera, although type 15 infections are reported to be uncommon (10, 12). Preliminary investigations have suggested that the precipitation could be caused by streptococcal IgG Fc-receptors in the extract (10). The frequency of precipitation varies between

populations, which indicates a restricted specificity for human IgG (10). It has hitherto not proved possible to separate M protein from the precipitating reactivity for human IgG (11).

It was recently found that Lancefield extracts of different types of group A streptococci – among them type 15 – have varying specificity for human red cells coated with various anti-Rh antibodies. The agglutination caused by the extracts was shown

to be due to an interaction between the extract and the Fc part of IgG (2).

We now describe that the Fc receptor of type 15 group A streptococci can be separated from M protein, peptidoglycan and group-specific carbohydrate by subjecting *alkaline* extracts of the bacteria to agarose gel electrophoresis.

MATERIALS AND METHODS

Streptococcal Strains

The following reference strains of group A streptococci were used: M 1 (8198), M 8 (8324), M 15 (100070) and the Griffith strain T 44 (Henson glossy).

Streptococcal Extracts

Group A streptococci type 15 were cultured in 20 litres of Todd Hewitt broth and harvested as described previously (10). The bacteria were washed in 0.15 M NaCl, suspended in 100 ml 0.15 M NaCl and the pH was raised to 10.0 by adding 2 M NaOH. The suspension was heated in a boiling water bath for 10 min and then immediately cooled on ice. The pH was adjusted to 7.0 with 2 M HCl, the suspension centrifuged at 3000 g for 15 min, and the supernatant used as alkaline extract.

Alkaline extracts of group A streptococci, types 1, 8 and T 44, were prepared from overnight cultures in 300 ml Todd Hewitt broth. The amounts of 0.15 M NaCl, NaOH and HCl used were reduced in proportion to the lesser volume of broth used.

The protein contents of the extracts as determined by a modified Folin method (6) were: M 1: 0.1 mg/ml, M 8: 1.3 mg/ml, M 15: 2.5 mg/ml and T 44: 1.6 mg/ml.

In some experiments, the alkaline extract of type 15 was trypsin-treated by addition of trypsin (Sigma, M.O., U.S.A.) to 2 mg/ml at pH 8.0. After incubation for 30 minutes at 37° C the trypsinization was stopped by addition of soy bean trypsin inhibitor (Sigma, type 1-s) to 2 mg/ml.

Peptidoglycan was prepared from M 1 group A streptococci with hot formamide, as described previously (9).

Human Sera, IgG, IgG Fragments and M-Components

Sera were collected from 23 apparently healthy individuals among the laboratory staff, heated at 56° C for 30 minutes, and kept at -20° C until use. Fab- and Fc-fragments of polyclonal human IgG were prepared as described earlier (2). Commercial polyclonal human IgG (batch no. 58803) was purchased from AB Kabi (Sweden) and used, unless otherwise indicated, at a concentration of 40 mg/ml phosphate-buffered saline (0.12 M NaCl, 0.05 M phosphate, pH 7.2).

The purified human myeloma proteins were identical with those used previously (2): 5 IgG1 M-components, 2 IgG2, 4 IgG3, 3 IgG4, 1 IgD and 3 IgM. Furthermore, one IgA M-component was purified as described earlier (2).

Electrophoretic Techniques

In the standard procedure for demonstration of precipitation lines between streptococcal extract and human serum, the streptococcal extract components were first separated by electrophoresis and the separated extract then tested against the serum by diffusion. Twenty ml 0.6% agarose (Miles Ltd., England) in 0.075 M barbital buffer, pH 8.6 containing 2 mM calcium lactate was poured over a 205 × 110 mm glass plate and circular wells taking 8 µl extract cut 75 mm from the projected anode. After application of the extract, a voltage of 200 V was applied for 45 min unless otherwise stated. A 2 mm broad channel, 90 mm long, was cut 3 mm from the application well, in the direction of the current. Two-hundred µl serum was applied and the diffusion was allowed to proceed for 2 days at room temperature.

Preparatory electrophoresis was performed in a 4 mm layer of 1% agarose in the barbital-lactate buffer. The extract was applied in a 6 mm broad channel, 190 mm long, cut in the agarose gel 75 mm from the projected anode. A voltage of 150 V was applied for 2 hours, after which the gel was cut into 5 mm wide slices. After freezing the pieces, overnight at -80° C followed by thawing and centrifugation at 3000 g, protein containing supernatants were harvested. The fractions were concentrated four times on a concentration cell (B15, Amicon corp., Mass., U.S.A.).

Electromigration experiments were performed in barbital-lactate buffer by 6 V/cm for 16 h with an agarose concentration of 1% (w/v) and 1.5 mg commercial polyclonal human IgG/ml gel.

The precipitates were stained with Coomassie Brilliant Blue.

Rabbit Anti-Streptococcal Sera

Rabbits were immunized intravenously with a suspension of washed and heat-killed streptococci, as described (8). Four animals were immunized with type 15 group A streptococci, and two each with types 1, 8, and T 44 streptococci. Anti-group A, C, and G rabbit sera were purchased from Difco (Mich., U.S.A.).

Agglutination-Tests with Ri-coated human red cells were performed as described previously (10).

RESULTS

Precipitation Patterns Observed in Diffusion Experiments Involving Electrophoretically Separated Alkaline Extract of Type 15 Group A Streptococci and Human Sera

After electrophoresis of the extract, three main precipitation archs were obtained in diffusion against commercial human polyclonal IgG (Fig 1): an anodal line (»1«), a double arch line extended along the well in which the extract was applied (»3«) and an elongated line situated near the serum well, extending from the cathodal side of the extract

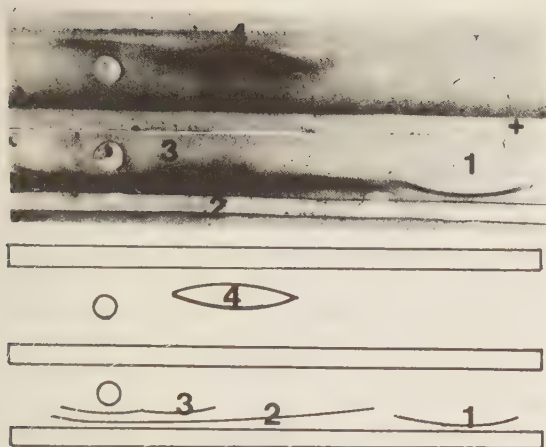


Fig. 1. Precipitation lines obtained in diffusion experiments involving electrophoretically separated alkaline extract of type 15 group A streptococci and human commercial polyclonal IgG or rabbit anti-type 15 serum. Upper trough: rabbit-anti type 15 serum; Lower trough: commercial polyclonal IgG; middle trough: saline. The round application wells for the alkaline extract are shown to the left. 1: Line 1; 2: line 2; 3: line 3; and 4: line 4 (see text). + indicates the anode.

application well to the cathodal start point of line 1 («2»).

Nineteen of 23 sera (83%) from apparently healthy individuals gave line 3, while 6 of them (26%) gave line 1. Line 2 was obtained with 2 sera only (9%). Lines 1 and 2 were not obtained with any of the sera without the presence of line 3. Four of the sera (17%) did not give any line.

Fifteen μ l of one of the sera giving both line 1 and line 3 (serum SM) was added to 5 μ l of the alkaline extract of type 15 and the mixture then subjected to electrophoresis. After diffusion against 5 sera giving both line 1 and line 3 with unabsorbed extract, no precipitation lines corresponding to 1 and 3 were obtained.

A few sera (2–6) gave precipitates corresponding to line 3 against the separated extracts of types 1, 8, and T 44 group A streptococci, but precipitates corresponding to line 1 were not seen. Line 2 was not obtained with types 1, 8 and T 44 extracts.

Separation of the Streptococcal Components Responsible for the Three Precipitates with Human Sera

In the following experiments the results obtained refer to tests with commercial human polyclonal IgG and a human serum giving both line 1 and line 3 (serum SM).

Lines 1 and 3 disappeared, while line 2 was unaltered after trypsinization of the type 15 alkaline

extract. Line 2 could be inhibited by addition of peptidoglycan to the commercial human IgG or serum SM.

Line 1 was obtained with the fractions precipitated at 20–30% ammonium sulphate saturation of the extract, while line 3 was obtained with the fractions prepared by 40% saturation.

Serum SM and commercial human IgG were absorbed with Cowan I staphylococci as described (1). After absorption, an essentially unaltered line 3 was observed, while line 1 had disappeared.

The electrophoretic fractions of the extract giving lines 1 and 3 were cut out of the agarose gel after electrophoresis. Addition of the «line 1 -fractions» to SM or to commercial human IgG inhibited only the formation of line 1, but not line 3, while the «line 3 -fractions» only suppressed line 3.

Localization of the M-Protein and Group A Carbohydrate after Electrophoresis of the Alkaline Extract of Type 15 Streptococci

All four anti-type 15 rabbit sera gave a line against the separated extract situated between the positions of line 1 and 3 observed with human sera (line 4, Fig. 1). Line 4 was not obtained between the anti-type 15 sera and the extracts of types 1, 8, and T 44 group A streptococci. Rabbit antisera to types 1, 8, and T 44 did not give line 4 with the alkaline extract of type 15. Line 4 disappeared after trypsinization of the extract.

Anti-group A carbohydrate serum gave a precipitate against a non-migrating component in the electrophoretically separated extracts of types 1, 8, 15, and T 44. This precipitate was not obtained with anti-C or anti-G rabbit sera. The precipitates were obtained also after trypsinization of the extracts.

Localization of the Streptococcal IgG Fc-Receptor after Electrophoresis of the Alkaline Extract of Type 15 Group A Streptococci

After electrophoresis of the extract, pieces of gel were cut out and investigated for content of agglutinating activity against Ri-coated human red cells. Only the fractions surrounding the application well, corresponding to line 3, agglutinated the cells (titres: 1:200–1:3200).

The alkaline extract was mixed with equal volumes of Fc-fragments or Fab-fragments of pooled human IgG at concentrations of 0.6–2.5 mg/ml. The mixture was subjected to electrophoresis and tested against serum SM and commercial human IgG in diffusion experiments. On addition of Fc-fragments to the extract, line 3 moved against the anode, whereas Fab-fragments did not cause any change in line 3 (Fig. 2).

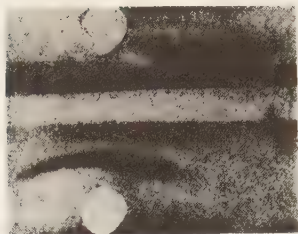


Fig. 2. The effect of addition of Fc-fragments of polyclonal IgG to alkaline extract of type 15 group A streptococci on the precipitation lines obtained between the electrophoretically separated extract and serum SM in diffusion experiments. Trough: serum SM; upper well: application well for equal volumes of Fc-fragments and alkaline extract; lower well: application well for alkaline extract diluted 1:2. + indicates the anode.

The alkaline extract mixed with Fc-fragments as described above was subjected to electrophoresis and tested by diffusion experiments against rabbit anti-group A carbohydrate serum. The precipitate obtained did not change its character or position as compared with the results obtained with the extract alone.

Characterization of Line 1

The streptococcal components responsible for line 1 were isolated by preparatory electrophoresis as described in «Methods». The preparation was then tested in electromigration experiments by applying it in a hole in an agarose gel in barbital-lactate buffer containing commercial human polyclonal IgG, which resulted in a «rocket» precipitate



Fig. 3. Electromigration of fractions from preparatory electrophoresis corresponding to line 1. Agarose gel containing 1.5 mg commercial human polyclonal IgG/ml was used. Left well: fraction corresponding to line 1, diluted 1:2. Right well: fraction corresponding to line 1, mixed with an equal volume of Fab-fragments (2.5 mg/ml) from human polyclonal IgG. Middle well: same as in the right well, but Fab fragments 1.2 mg/ml were used.

(Fig. 3). Mixing the preparation with equal volumes of Fc-fragments, 0.6 – 2.5 mg/ml, had no effect on the precipitate. Mixing the preparation with the same amount of Fab-fragments of pooled human IgG, however, resulted in depression of the precipitate (Fig. 3).

The preparation from the preparatory electrophoresis was also tested in the same system after mixing with 19 different purified human M-components at a concentration of 2 mg/ml. No alteration of the precipitate obtained with commercial human IgG was found.

DISCUSSION

After electrophoresis of the alkaline extract of type 15 group A streptococci it was possible to obtain strong precipitates in immunodiffusion experiments with sera from apparently healthy individuals. One of the precipitation lines obtained, line 3, was ascribed to a streptococcal Fc-receptor for human IgG, since the line could be displaced by addition of Fc-fragments, but not Fab-fragments of pooled human IgG. Fc-fragments migrate towards the anode in electrophoresis performed under the conditions described here (3). Furthermore, preparative electrophoresis of the alkaline extract showed that the fractions giving line 3 were capable of agglutinating IgG-coated human red cells at high titres. Line 3 was obtained with 19 of 23 human sera, 83%, a proportion close to the 82% Swedes found to precipitate hot hydrochloric acid extract of type 15 in gel diffusion experiments (10).

Line 4, obtained in diffusion experiments involving electrophoretically separated alkaline type 15 extract and rabbit anti-sera to type 15 group A streptococci was judged to contain streptococcal M-protein. It was only obtained with antisera to type 15, and not with antisera against 3 other types. The antisera to type 15 did not give such a line with extracts of other streptococci. Furthermore, the component responsible for the precipitate in the alkaline extract was, like M-protein, trypsin sensitive. Since the components responsible for lines 3 and 4 had differing electrophoretic mobility it is clear that the streptococcal Fc-receptor for human IgG in type 15 group A streptococci is separable from the precipitating, type-specific M-protein.

The streptococcal IgG Fc-receptor could also be separated from the group A carbohydrate, since the receptor was trypsin-sensitive and could be displaced by electrophoresis after addition of human IgG Fc-fragments, in contrast to group A carbohydrate. Also, the Fc-receptor was separable from peptidoglycan, since line 3 was not suppressed by addition

of peptidoglycan to the commercial human IgG, in contrast to line 2.

Six of the 18 sera giving line 3 gave also another line, line 1, against electrophoretically separated alkaline extract of type 15 group A streptococci. Line 1 was different from line 3, since line 1 was suppressed in contrast to line 3 on absorption of SM serum or commercial human IgG with Cowan I staphylococci; protein A of *S. aureus* reacts with 3 of 4 human IgG subclasses (5); with 1 of 2 IgA subclasses (7), and with about 30 % of human IgM (4). In electromigration experiments the component giving line 1 was altered by addition of Fab-fragments but not Fc-fragments of polyclonal human IgG, in contrast to the component giving line 3. It seems possible that line 1 is produced by an antigen-antibody precipitation, since myeloma proteins did not influence the component producing the line. The relation of the component giving line 1 to other streptococcal antigens – other than M-protein, peptidoglycan and the groupspecific carbohydrate – is still unknown. Type 15 infections in humans are seldom met (12), in contrast to type 1 group A streptococcal infections, for instance. This makes the frequent occurrence of line 1 as compared with the absence of such precipitates with the other types difficult to understand at present. The high protein concentration found in the type 15 extract as compared to the other extracts investigated might indicate that the type 15 extract could be »stronger« than the other preparations. However, the M1, M8 and T44 extracts concentrated 10 times did not give line 1 (unpublished observation).

The precise mechanism by which human IgG is precipitated by streptococcal Fc-receptors present in type 15 alkaline extract is also unknown. Preliminary studies have shown that the Fc-receptor also precipitates a number of purified human myeloma proteins. The finding that line 3 was displaced rather than suppressed, after mixing with Fc-fragments, might indicate that a larger part than the Fc-part of an IgG molecule is involved in the precipitation phenomenon. Furthermore, the double arch shape of line 3 regularly found might indicate that two or more Fc-receptors are present in the alkaline extract of type 15 group A streptococci. Purification experiments involving the Fc-receptor(s) in type 15 alkaline extract are in progress, and might elucidate the mechanism of precipitation.

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THE GROUP A STREPTOCOCCAL RECEPTOR FOR HUMAN IgA BINDS IgA VIA THE Fc-FRAGMENT

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Schalén, C. The group A streptococcal receptor for human IgA binds IgA via the Fc fragment. Acta path. microbiol. scand. Sect. C, 88: 271–274, 1980.

Eight freshly isolated type M4 strains of group A streptococci were found to bind between 60 and 80% of 2.5 µg radiolabelled IgA myeloma protein in a standard test system, while a reference type 4 strain bound only 20%. Commercial human IgG or IgG1 myeloma protein did not inhibit the binding of IgA by the reference type 4 strain or one of the freshly isolated type 4 strains, whereas inhibition was obtained by purified polyclonal IgA and IgA1 myeloma protein. Fc fragments of purified IgA1 myeloma protein, obtained by digestion with gonococcal protease, inhibited binding of radiolabelled IgA1, while the Fab fragments had no inhibitory effect.

Key words: Streptococci; human IgG; human IgA; Fab fragment; Fc fragment.

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Many group A, C and G streptococci have receptors for human IgG (1, 3, 4, 6, 7, 10), while IgA receptors have been demonstrated in only a few strains of group A streptococci (5, 10). *Christensen & Oxelius* (5) reported that the binding of IgA myeloma proteins to group A streptococci type 4 was not significantly inhibited by IgG and *vice versa*. Recently, separate receptors for human IgG and IgA were demonstrated in alkaline extract of type 4 group A streptococci (12).

The interaction between IgG and the corresponding streptococcal receptors has been localized to the Fc fragment of IgG (1, 3, 6). The present paper concerns the interaction between Fab and Fc fragments of IgA and the streptococcal IgA receptors. A recently described method for cleavage of IgA by gonococcal IgA protease was used to produce these IgA fragments (11).

MATERIALS AND METHODS

Bacterial Strains and Preparations

The group A streptococcal reference strains M4

(SS241) and M15 (EF 1949), and 8 freshly isolated type M4 strains were used in IgA-binding experiments. Standard suspensions of the strains were prepared as described by *Christensen & Oxelius* (4).

The gonococcal strain used for cleavage of IgA has been subcultivated five times a week for several years in our laboratory (strain 3K).

Immunoglobulin Preparations

Human purified polyclonal IgA, and one IgG1 and one IgA1 kappa-chain M-component were kindly supplied by Dr. A. Grubb, Malmö General Hospital, Malmö, Sweden. Human commercial IgG was purchased from AB Kabi, Sweden (batch No. 70972).

The IgA1 and IgG1 myeloma proteins were radiolabelled with ¹²⁵I (9) for detection of streptococcal IgA receptor and were also used in unlabelled state for inhibition studies.

Digestion of Monoclonal Human IgA1 by Gonococcal Protease

The gonococcal strain 3K was grown overnight at 37 °C in 6% CO₂ after heavy inoculation of two haematin agar plates (9 cm diameter). The colonies were harvested and suspended in 5 ml of IgA myeloma protein, 10 mg/ml in phosphate-buffered saline (PBS,

0.03 M phosphate, 0.12 M NaCl, pH 7.2). The mixture was incubated at 37 °C in rotating tubes for 17–18 hours, centrifuged at 10000 *g* and the supernatant sucked off and sterile filtered. The digested IgA myeloma protein was analyzed by immunoelectrophoresis (13).

Preparatory Electrophoresis

Preparatory electrophoresis was performed in a 4 mm layer of 1 % agarose (Miles Ltd, England) (in 0.075 M barbital buffer, pH 8.6, containing 2 mM calcium lactate), poured over a 205 × 110 mm glass plate (2). The crude digested IgA preparation was applied in a 6 mm broad channel, 190 mm long, cut in the agarose gel 75 mm from the projected anode. A voltage of 150 V was applied for 2 hours, after which the gel was cut into 5 mm wide slices. After freezing the pieces overnight at –80 °C followed by thawing and centrifugation at 3000 *g*, protein-containing supernatants were harvested.

Separation of Digested IgA M Component by Gel-Filtration

The digested IgA myeloma protein was separated on a Sephadex G200 column (2.5 × 90 cm; filtration in PBS at a rate of 18 ml/hour, 3 ml fractions).

Rabbit Antisera

Antiserum to IgA and antiserum to kappa chain were purchased from DAKO, Copenhagen, Denmark.

Protein Determinations

Protein concentrations were measured with a modification of Folin's method (8).

Determination of the Uptake of Radiolabelled IgA1 and IgG1 by Streptococci; Inhibition Experiments with Immunoglobulins and IgA fragments

The binding of radiolabelled IgA1 and IgG1 myeloma protein to the streptococci was measured as described by Christensen & Oxelius (4, 5) with slight modifications. An excess of streptococcal Ig receptors is allowed to react with a constant amount of radiolabelled Ig. PBS, 100 µl containing 0.1 % (vol/vol) Tween 20 (in order to minimize unspecific protein adherence), was mixed with 200 µl of the streptococcal standard suspension and 50 µl radiolabelled IgA1 or IgG1 (2.5 µg). After 30–45 min at 22 °C, 2 ml PBS containing 0.1 % Tween 20 was added, the tubes centrifuged at 3000 *g* and the supernatant sucked off. The radioactivity in the pellet was expressed as a percentage of the total amount of radioactivity added. In the inhibition experiments with different immunoglobulin preparations, the preparation to be investigated was first mixed with the streptococci and PBS containing 0.1 % Tween 20 and left at 22 °C for 2–5 min. After the addition of 50 µl radiolabelled IgA1 (2.5 µg), the binding of IgA1 to the streptococci was tested as described above.

RESULTS

Binding of Radiolabelled IgA1 and IgG1 by Some Group A Streptococci

All 8 freshly isolated type M4 strains investigated bound 60 to 80 % of the ¹²⁵I-labelled IgA1

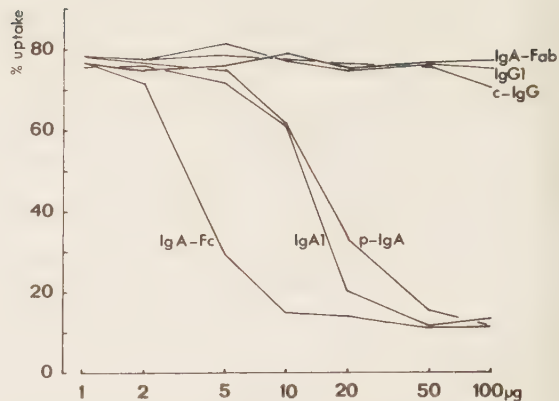


Fig. 1. The effect of addition of various unlabelled immunoglobulin preparations on the binding of radiolabelled IgA1 myeloma protein to type M4 streptococci, strain 595. Abscissa: µg of unlabelled immunoglobulin added. Ordinate: uptake of radiolabelled IgA1 myeloma protein in % of 2.5 µg added. IgA-Fab: Fab-fragments of IgA1; IgA-Fc: Fc-fragments of IgA1; IgA1: IgA1 myeloma protein; IgG1: IgG1 myeloma protein; c-IgG: commercial human IgG; and p-IgA: polyclonal IgA.

myeloma protein. In contrast, the reference strain of type M4, SS241 (which had been kept and subcultivated in the laboratory) showed an uptake of only 20 %. The uptake of type 15 group A streptococci was below 5 %. In the following experiments, two strains were used, the reference strain of type M4 and one freshly isolated M4 strain (595).

In order to investigate the specificity of the IgA binding, various unlabelled immunoglobulin prepa-

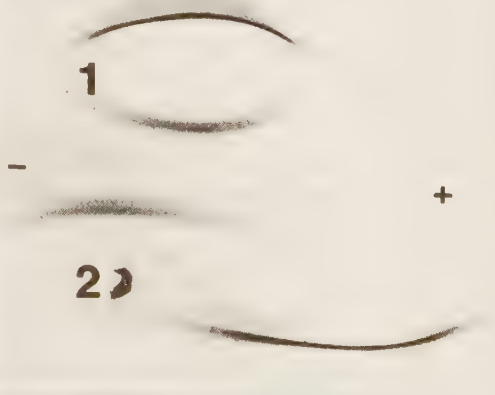


Fig. 2. Immunoelectrophoresis of digested (gonococcal protease) and undigested IgA1 myeloma protein. Well 1: application well for undigested IgA1. Well 2: application well for digested IgA1. Middle trough: anti-kappa serum. Upper and lower troughs: anti-IgA serum. + indicates anode.

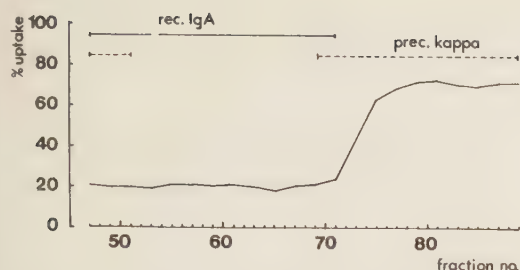


Fig. 3. The effect of addition of unlabelled fractions obtained from gel-filtration of gonococcal protease-digested IgA1 myeloma protein on the binding of radiolabelled IgA1 myeloma protein to type M4 streptococci, strain 595. Abscissa: indicates fraction No. Ordinate: uptake of radiolabelled IgA1 myeloma protein in % of 2.5 μ g added. Unbroken line indicates fractions precipitated by anti-IgA serum. Broken line: fractions precipitated by anti-kappa serum.

rations were tested for their capacity to inhibit the binding of radiolabelled IgA1 to the streptococci, using 1–100 μ g (200 μ l volumes) of the unlabelled compounds (Fig. 1). Increasing amounts of polyclonal IgA and IgA1 M component suppressed the uptake of IgA1 on strains 595 and SS241. On the other hand, no inhibition was found on addition of IgG1 M component or commercial human IgG.

The uptake of 125 I-labelled IgG1 myeloma protein by the 8 freshly isolated type M4 strains was between 7 and 45 (mean: 26) % of 2.5 μ g added, as compared to an uptake of 11 % shown by the M4 reference strain. There was no correlation between uptake of IgG1 and IgA1 in the single strain.

Test of the Capacity of IgA1 Fragments to Inhibit the Binding of IgA1 to Type 4 Group A Streptococci

Digested IgA1 separated by gel filtration. IgA1 myeloma protein, 50 mg, was digested with gonococcal protease. Fig. 2 shows an immunoelectrophoresis of digested and undigested IgA1 M component involving anti-IgA and anti-kappa serum. The digested IgA showed an anodal displacement of the precipitate obtained with anti-IgA as compared with undigested IgA, while the precipitate given by anti-kappa serum had moved slightly cathodally. The digested protein was filtered on a Sephadex G200 column and 500 μ l of each fraction was examined for capacity to inhibit the binding of radiolabelled monoclonal IgA1 by type 4/595 (Fig. 3) and SS241. Fractions 41–71 precipitated with anti-IgA serum in double diffusion-in-gel. Fractions 45–51 and 69–89 precipitated with anti-kappa serum. Only the fractions precipitated by anti-IgA serum inhibited the binding of radiolabelled IgA1 to the two type 4 strains (Fig. 3).

From the results of molecular weight determinations on calibrated Sephadex G200 column (performed by Dr. A. Grubb, Malmö) it was concluded that the fractions 53–67 were aggregated Fc fragments, while fractions 73–89 represented Fab fragments (Fig. 3). The Fab and Fc fragments were pooled separately. A decrease in binding of radiolabelled IgA1 myeloma protein to strains 595 (Fig. 1) and SS241 was found on addition of increasing amounts of Fc fragments, while similar amounts of Fab fragments had no effect.

Digested IgA1 separated by preparatory electrophoresis. The digested IgA1 myeloma protein, 10 mg,

TABLE 1. Inhibition Studies on the Uptake of Radiolabelled IgA Myeloma Protein on Type 4 Group A Streptococci (Strains 595 and SS241) Using Fractions Obtained from Preparatory Electrophoresis of Digested IgA Myeloma Protein

Fraction no.	Precipitated in double diffusion-in gel by		Addition of the fraction: effect on uptake of radiolabelled IgA (in %) by	
	anti-IgA serum	anti-kappa serum	strain SS241	strain 595
2	—	—	17	73
3	—	—	17	73
4	—	—	17	75
5	—	+	16	73
6	+	+	3	16
7	+	+	2	15
8	+	—	5	37
9	+	—	4	48
10	—	—	16	74
11	—	—	16	73
12	—	—	17	73

was subjected to preparatory electrophoresis. All fractions (200 µl) that precipitated with anti-IgA inhibited, as also the anodal fractions 8 and 9 (Table 1), which did not precipitate with anti-kappa serum. No inhibition was obtained with fraction 5, which precipitated with anti-kappa but not with anti-IgA.

DISCUSSION

Receptors for human IgA on group A streptococci were shown in 1975 by Christensen & Oxelius (5). The IgA receptor of type 4 streptococci differed from the IgG receptor present on this streptococcal type as shown in experiments with uptake on whole bacteria of radiolabelled immunoglobulins (5) and with solubilized receptors (12). Group A streptococcal IgA receptors have affinity for both subclasses of human IgA (10).

The present results demonstrated that all of 8 tested freshly isolated type 4 group A streptococci bound between 60 and 80% of radiolabelled IgA1 M component, in contrast to the reference strain type M4 SS241, with an uptake of about 20%. The SS241 strain had been subcultivated and kept for several years in the laboratory. In inhibition experiments using polyclonal IgA and IgG as well as IgA and IgG M components, only IgA was found to inhibit the binding, which tallies with earlier results (5, 10).

Gonococci were able to digest monoclonal IgA1, which confirms the findings by Plaut *et al.* (11). The electrophoretic fractions containing IgA Fc fragments strongly inhibited the uptake of undigested radiolabelled IgA in the two type 4 strains tested, while the fractions containing Fab but not Fc fragments were non-inhibitory. Similar results were obtained using fractions from gel-filtration experiments of digested IgA myeloma protein. Thus, the data clearly demonstrated that IgA interacts with the streptococcal receptor via the Fc fragment.

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DEMONSTRATION OF SEPARATE RECEPTORS FOR HUMAN IgA AND IgG IN GROUP A STREPTOCOCCI TYPE 4

*Separation of the Solubilized Receptors from Group- and Type-Specific Antigens,
Lipoteichoic Acid and Peptidoglycan*

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Schalén, C., Christensen, P., Grubb, A., Samuelsson, G. & Svensson, M.-L. Demonstration of separate receptors for human IgA and IgG in group A streptococci type 4. Separation of the solubilized receptors from group- and type-specific antigens, lipoteichoic acid and peptidoglycan. Acta path. microbiol. scand. Sect. C, 88: 77-82, 1980.

The alkaline extract of group A streptococci type 4 was separated by electrophoresis and diffused against 27 normal human sera. One of the precipitates appeared with 85% of the sera. Addition of purified IgA myeloma protein or sera containing IgA M-components to the extract changed the electrophoretic mobility of the precipitate anodically. Purified IgG Fc-fragments or sera containing IgG M-component did not affect the mobility of the precipitate. It was concluded that this precipitate contained the streptococcal receptor for human IgA. A non-precipitating IgG Fc-receptor, with agglutinating capacity for cells coated with human IgG1 but not IgG3, was localized by preparative electrophoresis to the same electrophoretic region as the IgA receptor. The mobility of the IgG receptor remained unaltered on addition of IgA myeloma protein permitting a separation of the two receptors by preparative electrophoresis. The receptors were distinct from the group specific carbohydrate, peptidoglycan and lipoteichoic acid. No M antigen or opacity factor were demonstrated in the extract.

Key words: Streptococci; M protein; peptidoglycan; group A carbohydrate; lipoteichoic acid; human IgA; human IgG.

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Most streptococci belonging to groups A, C and G contain receptors for the Fc-part of human IgG (1, 3). The receptors can be released from the cells by hot hydrochloric acid or alkali extraction (1, 2, 14). The solubilized receptors of different types of group A streptococci have varying affinity for human IgG1 and IgG3 in agglutination experiments with human red cells coated with various anti-Rh antibodies (1). By use of diffusion-in-gel, it was shown that the Fc receptor for human IgG from group A streptococci type 15 was precipitated by

83% of normal Swedish sera and that it was distinct from the M protein, peptidoglycan and group-specific carbohydrate by electrophoresis (2).

The binding of radiolabelled IgA myeloma protein to some group A streptococci was described in 1975 (4). The data obtained indicated the existence of structures with high affinity for IgA on different streptococcal strains and that radiolabelled IgG and IgA were bound to the streptococci independently. Among the reference strains of group A streptococci tested a type 4 culture showed an outstanding capacity for binding of IgA. Later,

group A and G streptococci, containing T-type 4 antigen were found to agglutinate in highly diluted sera containing haptoglobin of types 2-1 and 2-2 but not of 1-1 (5, 12).

We now report that group A streptococci possess two separable immunoglobulin receptors, one for IgA and one for IgG. Furthermore, the relationship of the receptors to some other streptococcal factors was investigated, such as group- and type-specific antigens, peptidoglycan, haptoglobin-binding activity and lipoteichoic acid.

MATERIALS AND METHODS

Streptococcal Preparations

Alkaline extracts of types 4 (strain SS 241) (alk. ex. T4) and 15 (strain 100070) (alk. ex. T15) group A streptococci were prepared from overnight cultures in 20 litres of Todd Hewitt broth as described (2). Several batches prepared on different occasions were studied. The protein content of the type 4 extracts as determined by a modified Folin method (8) was 0.5 to 0.9 mg/ml. The line 1 components in the alk. ex. T15 earlier described were isolated by preparative electrophoresis (2). The protein content of this fraction was 1.3 mg/ml.

In some experiments alk. ex. T4 was treated by trypsin as described (2). Peptidoglycan was prepared from type 1 group A streptococci (strain 8198) with hot formamide (13). Purified lipoteichoic acid (LTA) from *S. mutans* was kindly supplied by Dr. I. Ginsburg, Jerusalem, Israel.

Human Sera, M-Components, IgG Fc-Fragments and Haptoglobin

Sera were collected from 27 apparently healthy individuals among the laboratory staff. Seventeen myeloma sera, 9 containing IgG M-component (IgG content 12 to 65 mg/ml) and 8 IgA M-component (IgA content 19 to 34 mg/ml) were used. The concentration of polyclonal Ig in all sera was below 1 mg/ml. The purified human myeloma proteins were identical with those previously used (1, 2). Fc-fragments of polyclonal human IgG were prepared as earlier described (1). Three sera of haptoglobin type 1-1, 3 of type 2-1 and 4 of type 2-2 were randomly selected. Haptoglobin-typing was performed as described by Laurell (7). Haptoglobin type 2-2 purified as described by Laurell (6) was available at the laboratories.

All sera were heated at 56 °C for 30 min and stored at -20 °C until used.

Electrophoretic Techniques

Precipitates between electrophoretically separated streptococcal extracts and human sera were done as described previously (2). In brief, the streptococcal extract components were separated by electrophoresis and the separated extract then diffused against serum, applied in a channel cut in the gel in the direction of the current. The precipitates obtained between extract of type

4 group A streptococci and human sera were designated 1_{T4}, 2_{T4} etc.

Preparative electrophoresis was performed as described (2).

Immunoelectrophoresis of human sera containing M-component was performed using rabbit antisera to IgA and IgG (Behringwerke, West Germany), 1-2 µl of serum diluted 1:20 was applied and the antisera were used in 1:5 dilutions.

Rabbit Anti-Streptococcal Sera

Antiserum for M-typing of group A streptococci type 4 was kindly supplied by Dr. A. El Kholi, Cairo, Egypt. Rabbit anti-group A, C and G sera were purchased from Difco (Michigan, U.S.A.) Anti-group A streptococcus serum, cross-reacting with *S. mutans* lipoteichoic acid was kindly supplied by Dr. I. Ginsburg.

Test for Streptococcal Opacity Factor (OF)

The presence or absence of OF in streptococcal extract was investigated by the plate method described by Maxted *et al.* (9).

Test for Streptococcal Lipoteichoic Acid

The test for the presence of LTA in the streptococcal extract was performed essentially as described by Ofek *et al.* (10). Human group 0, Rh-negative red blood cells, suspended to 2% (vol/vol) in saline, were treated with 100 µg/ml of papain in the presence of 10 µg/ml of cysteine for 30 min at 37 °C. The sediment from 0.5 ml of the treated red cells was suspended in 0.5 ml of the fractions to be tested and the mixtures incubated at 37 °C for 30 min. After three washings in saline, a 2% suspension of the cells was again prepared. The cells were then tested for agglutination in 0.2 ml volumes of twofold dilutions of anti-LTA serum. The amount of LTA in the streptococcal extract was given as the agglutination titre obtained with the antiserum. The presence of LTA in the extract preparations was further confirmed in inhibition studies using papain-treated human red cells coated with purified *S. mutans* LTA (10).

Hemagglutination and Hemagglutination Inhibition (HAI) Studies

These tests were performed as described previously, using the human anti-Rh antibodies Ri, KM and HUN (1, 14).

RESULTS

Precipitation Patterns of Electrophoretically Separated Alkaline Extract of Type 4 Group A Streptococci with Normal Human Sera

After electrophoresis of the extract, four main precipitation arches were obtained in diffusion against the sera from 27 apparently healthy individuals (precipitates 1_{T4}, 2_{T4}, 3_{T4} and 4_{T4}), (Fig. 1). Twenty-three of 27 sera (85%) gave the 3_{T4} precipitate, while 7 of them (26%) gave 1_{T4}, and 5 (19%) 4_{T4}. The precipitate 2_{T4} appeared with only

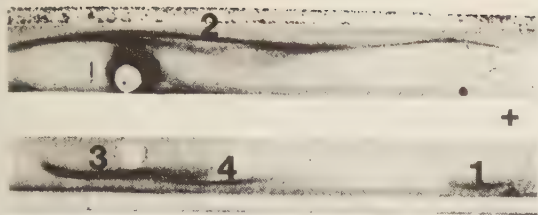


Fig. 1. Precipitation pattern on diffusion of electrophoretically separated alkaline extract of type 4 group A streptococci against two different human sera, applied in the troughs. The precipitates appearing are nominated 1, 2, 3 and 4, respectively. Anode to the right.

one serum. Precipitates 1 and 2 appeared only in sera containing also precipitate 3. The precipitate 4_{T4} was obtained with two sera which did not give 3_{T4}.

Ten μ l of one of the sera with precipitates 1_{T4}, 3_{T4} and 4_{T4} (serum KP) was added to 10 μ l of the type 4 extract and the mixture subjected to electrophoresis. On diffusion against 6 sera the precipitates corresponding to 1_{T4}, 3_{T4} or 4_{T4} were no longer obtained.

Electrophoretic Mobility of the Streptococcal Receptor for IgA of the Alkaline Extract of Type 4 Group A Streptococci

The alkaline extract was mixed with equal volumes of human sera containing high amounts of IgG (8 sera) or IgA (9 sera) M-components. After electrophoresis of the mixtures containing IgA M-component and diffusion against the normal serum KP, 3_{T4} had moved anodally (Fig. 2). In contrast, 7 of the sera with IgG M-component did not affect the precipitate 3_{T4} while with one IgG M-component serum the precipitate 3_{T4} was no longer visible.

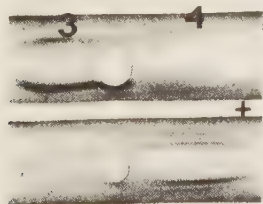


Fig. 2. The effect of addition of purified IgA myeloma protein (2 mg/ml) to the alkaline extract of type 4 group A streptococci on the precipitates obtained between the electrophoretically separated extract and serum KP in diffusion experiment. Note that precipitate 3_{T4} is shifted anodally while 4_{T4} remains in the same position. Troughs: normal serum KP; lower well: application for equal volumes of IgA myeloma protein and alkaline extract; upper well: application for alkaline extract diluted 1:2; + indicates the anode.

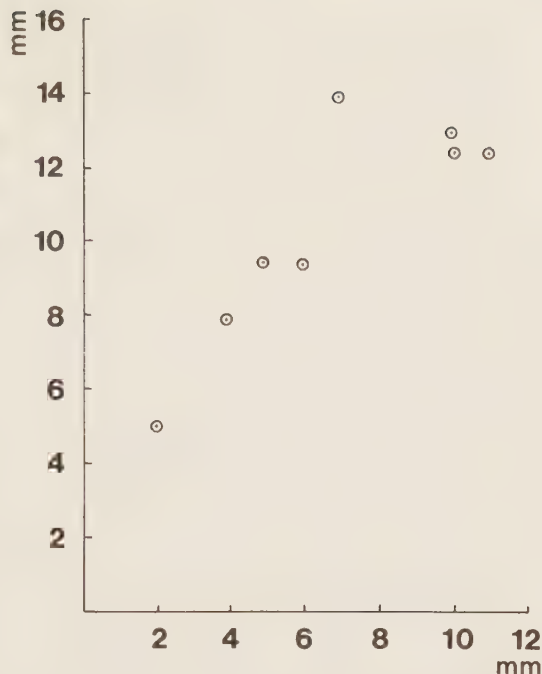


Fig. 3. Dislocation of the 3_{T4} precipitate obtained between the type 4 extract and serum KP on addition of human sera containing IgA M-component to the extract set in relation to the electrophoretic mobility of the IgA M-components. The position of each precipitate was defined as the point on the arch situated closest to the serum channel. Ordinate: Distance between the localization of the IgA M-component of each serum and the serum application well in immunoelectrophoresis. Abscissa: anodal displacement of 3_{T4} by addition of the corresponding serum to the alkaline extract before electrophoresis.

The sera with IgA M-component were tested in immunoelectrophoresis with anti-IgA serum. All M-components were localized anodally in relation to the application well. The dislocation of the precipitate 3_{T4} and the electrophoretic charge of the IgA M-components used in the individual experiment correlated ($r = 0.82$).

Immunoelectrophoresis of the sera containing IgG M-component revealed that 5 of the M-components were situated anodally and 3 cathodally in relation to the application well.

Addition of equal volumes of the solution of isolated IgA myeloma protein (2mg/ml) to the extract moved 3_{T4} towards the anode while IgG Fc-fragments (0.6–2.5 mg/ml) did not affect line 3_{T4} or any of the other precipitates.

Separation of the Streptococcal Receptor for IgG from the IgA Receptor

After electrophoresis of the extract, pieces of gel

were cut out and investigated for content of agglutinating activity against anti-Rh(Ri)-coated human red cells (1). Only the fractions surrounding the application well, corresponding to the precipitate 3_{T4}, were found to agglutinate the cells (titres 1:100–1:1600).

The fractions of alk. ex. T4 corresponding to precipitate 3_{T4} were pooled and tested for agglutinating capacity against cells coated with various anti-Rh antibodies. Cells with the coat KM (diluted 1:5 at coating), useful for detection of G1m(1), were agglutinated by the fractions diluted 1:40, while cells with two different G3m(5) coats were not agglutinated.

Various purified IgG M-components were tested for their capacity to inhibit the agglutination of human red cells coated with KM by the fractions corresponding to the 3_{T4} precipitate. The fractions were used in dilution 1:10. An IgG1 M-component and an IgG3 M-component were both capable of inhibiting at a concentration of 0.1 mg/ml. Purified IgG Fc-fragments, 0.05 mg/ml, also inhibited the agglutination. A purified IgA M-component was not inhibitory at a concentration of 2 mg/ml.

A preparative electrophoresis was run with alk. ex. T4 after addition of an equal volume of a solution of a purified IgA M-component (2 mg/ml); as described above, the components responsible for line 3_{T4} were thus shifted towards the anode. The distribution of the agglutinating capacity for Ri-coated human red cells in the different fractions was not changed.

Lack of M-Antigen and Opacity Factor in the Alkaline Extract of Type 4 Group A Streptococci

No precipitation was obtained in double diffusion-in-gel experiments between the alk. ex. of T4 and anti-type 4 M-typing serum. No opacity factor activity was demonstrable in the extract.

Comparison of the Components Responsible for Precipitate 1 in the Alkaline Extracts of Types 4 and 15 Group A Streptococci

The 7 sera giving precipitate 1_{T4} with the type 4 alkaline extract also precipitated the fractions corresponding to line 1 in the alk. ex. T15. The electrophoretic fractions corresponding to the precipitate 1_{T4} were isolated by preparative electrophoresis. Ouchterlony type 1 reactions (interference with complete fusion (11)) were obtained on double diffusion-in-gel experiments between the components responsible for line 1 in the alk. ex. T15 and the precipitate 1_{T4}.

Localization of Group A Carbohydrate, Lipoteichoic Acid and Peptidoglycan after Electrophoresis of the Alkaline Extract of Type 4 Group A Streptococci

Anti-group A carbohydrate serum gave a precipitate against a component in the electrophoretically separated extract situated immediately anodally to the application well. This precipitate was obtained also after trypsinization of the extract. Anti-C and anti-G sera gave no such line.

Precipitates 1_{T4}, 3_{T4} and 4_{T4} disappeared, while line 2_{T4} remained unaltered after trypsinization of alk. ex. T4. 2_{T4} could be inhibited by addition of peptidoglycan to the serum causing the 2_{T4} precipitate.

Papain-treated human red blood cells were coated with the fractions obtained by preparative electrophoresis of alk. ex. T4. Except the material from the 2 slices taken most cathodally, all fractions were capable to coat the red cells for agglutination by anti-LTA serum in dilutions from 1:20 to 1:320. In addition, these fractions could inhibit the agglutination of cells coated with purified LTA caused by anti-LTA serum.

Lack of Relation between the Receptors for IgA and IgG and Haptoglobin-Binding Capacity

Nine different sera belonging to haptoglobin types 1-1, 2-1 or 2-2 were tested by diffusion-in-gel against the electrophoretically separated alk. ex. T4. There was no relation between precipitates 1_{T4}, 2_{T4}, 3_{T4} and 4_{T4} and the haptoglobin type.

Addition of solutions of purified haptoglobin of type 2-2, 1.5–48.4 mg/ml, in equal volumes to the alk. ex. T4 did not affect the appearance of any of the 1_{T4}, 2_{T4}, 3_{T4} and 4_{T4} precipitates.

The agglutination of KM-coated human red blood cells by the fractions from a preparative electrophoresis of alk. ex. T4 was not inhibited by purified haptoglobin type 2-2 in concentrations of 1.5–48.4 mg/ml.

DISCUSSION

Electrophoretic separation of the alkaline extract of type 4 group A streptococci gave rise to 4 well separated precipitates in gel-diffusion experiments with sera from healthy individuals. The location of the precipitates, and the frequencies with which they occurred were similar to the results obtained with an alk. ex. T15 described earlier (2), with the exception of the 4_{T4} precipitate which was only demonstrable in experiments with type 4 extract. The 4_{T4} precipitate was not further characterized in the present work.

The component in the type 4 extract responsible

for 3T₄ resembled the IgG Fc-receptor shown in the type 15 extract (2) with respect to electrophoretic mobility and the frequency by which human sera were precipitated. However, the component from type 4 was apparently different from the type 15 IgG Fc-receptor, since the latter is shifted anodally in electrophoresis on addition of IgG Fc-fragments (2) in contrast to the component of 3T₄ precipitate. On the other hand, the mobility of 3T₄ shifted anodally in the presence of IgA M-components, while IgA M-components did not influence the line 3 of type 15 (unpublished observation). Evidently, the type 4 component responsible for line 3T₄ interacted with IgA independently of the specificity of the antibody combining sites, since 9 different IgA M-components caused a shift of the precipitate to the anode. This finding indicates that the type 4 component responsible for 3T₄ represents the earlier described streptococcal receptor for human IgA (4). It is not known whether the IgA receptor is active for both IgA subclasses and secretory IgA.

In the fractions corresponding to the 3T₄ precipitate an agglutinating capacity for anti-Rh coated human red blood cells was also demonstrated. These fractions agglutinated cells coated with IgG1 subclass antibodies, but not with IgG3 antibodies. The lack of reactivity at the sensitivity level used for IgG3-coats distinguishes the type 4 extract from that of type 15 (1); another difference is that the IgG receptor of type 15 is easily precipitated by human normal sera, which is not the case with the type 4 IgG receptor. A certain reactivity of the type 4 extract for IgG3 was demonstrable, however, since IgG3 (as also IgG1) myeloma protein was inhibitory in the HAI experiments. Fc-fragments of polyclonal human IgG were also inhibitory, while the isolated IgA myeloma protein was not. The IgA receptor precipitate shifted to a more anodal position on addition of IgA myeloma protein to the extract, while the agglutinating activity for IgG-coated cells remained in the fractions at the application well. This finding shows that two different receptors for human immunoglobulins – one for IgG and one for IgA – were present in the alkaline extract of type 4 group A streptococci. Only the IgA receptor was precipitated by human sera.

The precise mechanism by which 85 % of normal human sera precipitate the IgA receptor is at present unknown. The 3T₄ precipitate was changed as to mobility rather than suppressed on addition of IgA-components. On the other hand, serum KP suppressed the 3T₄ precipitate. This difference between IgA M-component sera and normal serum might reflect differences in the molecular structure of IgA or might be caused by additional factors present in the normal serum but absent from IgA-

component sera. The precipitation mechanism is at present under investigation.

The 2T₄ precipitate was – as in the experiments with type 15 extract (2) – caused by peptidoglycan. This precipitate did not appear after addition of peptidoglycan to the human serum. The components responsible for precipitate 1 in both type 4 and type 15 alkaline extract were antigenically identical as demonstrated by double diffusion-in-gel.

The absence of type-specific antigens in the alkaline extract of type 4 group A streptococci and the presence of receptors for IgA and IgG were in accordance with the observation of non-identity between these components earlier made (2). Also the immunoglobulin receptors differed in electrophoretic mobility and trypsin-sensitivity from peptidoglycan and group-specific carbohydrate. In contrast to the Ig receptors LTA was found in nearly all fractions obtained from preparative electrophoresis of type 4 alkaline extract.

This investigation has shown that group A streptococci type 4 possess distinct, well separable receptors for human IgA and IgG.

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Isolation and Some Properties of an IgG Fc-Binding Protein from Group A Streptococci Type 15

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Abstract. An IgG Fc-binding protein was isolated from alkaline extracts of group A streptococci type 15 by ion-exchange chromatography and immunosorption on an IgG column. Ample use of protease inhibitors was necessary to achieve successful isolation. 600 µg protein was obtained from 60 g bacteria (wet weight). The protein appeared homogeneous on agarose gel and sodium dodecyl sulfate polyacrylamide gel electrophoresis and had an apparent molecular weight of 29,500. It contained appreciable amounts of the amino acids glutamic acid, alanine, leucine, aspartic acid and lysine, but little or no tyrosine, phenylalanine, proline, glucosamine or galactosamine. It precipitated human monoclonal IgG of all four sub-classes in agarose gels as well as polyclonal IgG, IgG Fc and normal human serum. It did not precipitate IgG Fab, IgA, IgM, IgD or free κ or λ chains.

Introduction

Microorganisms may interact with immunoglobulins in two principally different ways. In the first way, the antigen-antibody reaction in a restricted sense, the antigen-combining sites of the immunoglobulin molecules participate, while in the second way, other parts of the molecules are involved. Although the known examples of the first type of interactions far outnumber the known cases of the second type, several recent reports have appeared which demonstrate interactions also of the second type. Thus, interactions of this type with IgG [8, 17], IgA [6, 26] and IgD [7] have been described. The only microbial substance interacting in this way with immunoglobulin which has so far been purified to homogeneity and characterized is protein A from *Staphylococcus aureus* [9]. This work describes the isolation from group A streptococci type 15 of a substance which binds to IgG molecules at areas outside the antigen-combining sites. It also reports certain of the physicochemical and immunochemical properties of the substance which appears to be a protein.

Materials and Methods

Dextran T10 and CNBr-activated Sepharose 4B were obtained from Pharmacia, Uppsala, Sweden, Ultrogel AcA44 from LKB, Bromma, Sweden, DEAE-cellulose DE52 from Whatman, Balston, England, Trypsin-TPCK from Worthington Biochemical Corp., Freehold, N.J., agarose (ME) from Marine Colloids, Rockland, Me., Todd-Hewitt broth from Difco Laboratories, Detroit, Mich., sodium heparinate from Kabi AB, Stockholm, Sweden, and diisopropylfluorophosphate (DIFP) and phenylmethylsulfonyl fluoride (PMSF) from Sigma, St. Louis, Mo. All other chemicals were of reagent grade and obtained from British Drug Houses, Ltd, Poole, England.

Immunoglobulin Preparations. IgG M components of all sub-classes, IgA-, IgM and IgD M components were purified from plasma samples from patients with multiple myeloma. Salt precipitation, ion exchange and gel chromatography and preparative agarose gel electrophoresis [6] were used in the purifications. The purity of the M components was assessed by analytical agarose gel electrophoresis [14] and immunoelectrophoresis [27] and was in all cases estimated to be 95% or higher.

The subclasses of the IgG M components were determined by the chemical procedure described by Frangione et al. [9]. Polyclonal IgG and its Fc and Fab fragments were produced as described earlier [3]. (Fc)₂ μ fragment was prepared from an IgM M component by tryptic digestion at 56°C as reported by Plaut and Tomasi [24].

Affinity Chromatography. Polyclonal or monoclonal human

IgG was covalently attached to CNBr-activated Sepharose 4B according to the manufacturer's instructions. The gel was washed as recommended and in addition with several volumes of 0.1 M glycine buffer, pH 2.2, with 0.5 M NaCl, 1 mM iodoacetic acid and 1 mM benzamidine chloride.

Double Radial Diffusion. Holes were cut in agarose gels as for regular Ouchterlony analysis, but a 1% agarose gel in 0.05 M Tris buffer, pH 7.4, containing 10% (w/v) Dextran T10 and 200 IU heparinate/ml was used. The bacterial solutions were allowed to diffuse against different Ig solutions for 16 h at 4°C. The gel slabs were then washed in 0.05 M Tris buffer, pH 7.4, for about 24 h at room temperature, dried and stained with Kenacid blue R.

Single Radial Diffusion for Quantitation of IgG-Precipitating Capacity. A pure IgG M component was incorporated in a 1% agarose gel at a concentration of 15 µg/ml 0.05 M Tris buffer, pH 7.4, containing 10% (w/v) Dextran T10 and 200 IU heparinate/ml was used, for the gel and holes were cut in the gel slab as for single radial immunodiffusion, 8 µl of the various solutions of the IgG-binding substance was distributed into the holes and diffused for 3 days at 4°C. The gel slabs were then washed in 0.05 M Tris buffer for 1 day at room temperature, dried and stained with Kenacid blue R.

Immunochemical Quantitations. Standard methods were used for electroimmunoassay [20], single [22] and double [23] radial immunodiffusion.

Sodium Dodecyl Sulfate (SDS) Polyacrylamide and Agarose Gel Electrophoresis. The procedures described by Laemmli [19] and Johansson [14] were used.

Amino Acid Analysis. Aliquots of various solutions of the isolated IgG binding substance in 0.05 M Tris buffer, pH 7.4, with 1 mM iodoacetic and 1 mM benzamidine chloride were evaporated in hydrolysis tubes. After addition of 6 M HCl the tubes were alternately pressurized with ultrapure argon and evacuated in four successive cycles and subsequently sealed under argon pressure and heated at 110°C for 20 h. The hydrolysates were analyzed by use of a single-column system on a Kontron Liquimat III amino acid analyzer with the Durrum DC-6A resin developed according to a program for physiological fluids. When the Tris buffer alone was hydrolyzed and analyzed as stated above, only one ninhydrin-positive peak appeared between, and well separated from, the methionine and isoleucine peaks.

Tests for Type 15 M Protein. The possible presence of streptococcal type 15 M protein in various solutions was tested by double radial immunodiffusion with use of three different anti-type 15 antisera. Two antisera were raised in rabbits as previously described [3], while another was kindly supplied by Dr. A.M. El Kholi, Cairo, Egypt.

Hemagglutination Tests. Human Rh-positive red blood cells were coated with 'incomplete' anti-Rh antibodies from various individuals. The capacity of different solutions to agglutinate the coated cells was tested in microtiter plates. The anti-Rh Ripley (Ri), exceptional by being complement binding and not limited to one subclass, was a gift from Dr. Marion Waller, Richmond, Calif. Anti-Rh KM is useful for detection of the human IgG1 genetic marker (Glm(1) and the anti-Rh HUN for the IgG3 marker G3m(5) [for details see 3].

Bacterial Cultivation and Extraction. Group A streptococci, type 15 (EF 1949) were cultured for 14–16 h at 37°C using magnetic stirrers, in 12×5 liters of Todd-Hewitt broth buffered with 0.02 M Tris buffer, pH 8.0. After culture, the purity of the bacteria was checked. Benzamidine chloride and iodoacetic acid were added

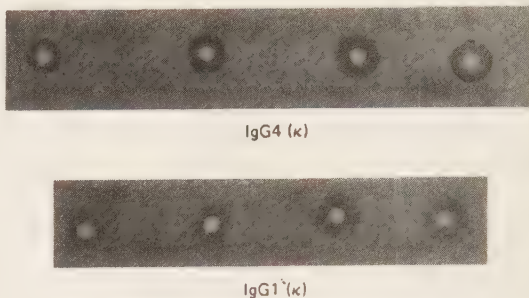


Fig. 1. Single radial diffusion of three solutions (100, 135 and 200 µg/ml) of the purified IgG-binding substance in agarose gel slabs containing 15 µg/ml of an isolated IgG4 (κ) (above) or IgG1(κ) (below) M component. A solution of protein A (50 µg/ml) was tested for comparison (far right).

to the broth cultures to final concentrations of 1 mM and the bacteria were then heat-killed at 70°C for 1 h, followed by a sterility check on blood-agar plates. The streptococci were harvested in a continuous action rotor in a Hispin 21 centrifuge (MSE, Sussex, England) at 5,000 g. The sediment was washed twice in 0.15 M saline containing 1 mM benzamidine chloride and 1 mM iodoacetic acid and suspended in 100 ml of the same solution. After addition of 200 µl of 2 M DIFP in dry isopropanol and 100 µl of 0.5 M PMSF in methanol the pH of the suspension was raised to 10.0 with 5 M NaOH. The suspension was heated in a boiling water bath for 10 min, and subsequently cooled, after which 200 µl of 2 M DIFP and 100 µl of 0.5 M PMSF was again added. The pH was adjusted to 7.0 with 5 M HCl, and the bacterial debris removed by centrifugation at 3,000 g. When the extract was tested by agarose gel electrophoresis followed by diffusion of the dispersed substances against a trough with normal human serum as earlier described [4], a single strong precipitation line was obtained. This precipitate was displaced anodally when human IgG Fc fragments were added to the bacterial extract before electrophoresis.

Results

Quantitation of the IgG-Binding Substance. When a solution of the isolated IgG-binding substance was diffused in agarose gel slabs containing different pure IgG M components, well-demarcated circular precipitation areas were produced (fig. 1). The size of the area was found to be directly proportional to the concentration of the IgG binder. This procedure was therefore used to follow the IgG binder during its isolation and to quantitate the yield of it in the various purification steps.

Isolation of the IgG-Binding Substance. The bacterial extract was concentrated to about 50 ml by pres-

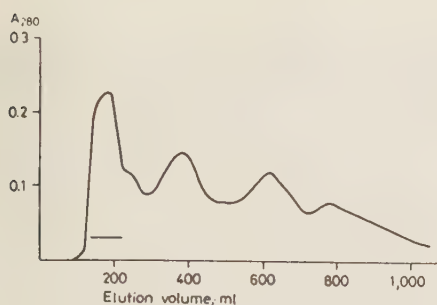


Fig. 2. DEAE-cellulose chromatography of concentrated bacterial extract. The fractions marked by a horizontal line precipitated an isolated IgG M component in single radial diffusion and were pooled.

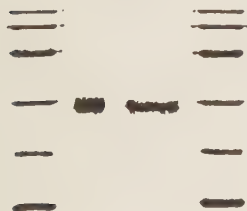


Fig. 3. SDS polyacrylamide gel electrophoresis of native (right) and reduced (left) IgG-binding protein. 6 μ g peptide material was boiled for 5 min in a buffer containing 2% SDS with or without 5% mercaptoethanol and subsequently applied to a 10% gel slab. A reduced molecular weight marker solution was simultaneously analyzed (far right and far left). The molecular weights of the markers were: 94,000, 67,000, 43,000, 30,000, 20,000 and 14,400.

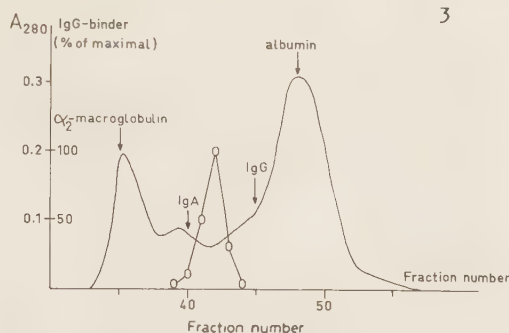


Fig. 4. Chromatography of the purified IgG-binding substance on a column of AcA44 calibrated with human plasma proteins of known molecular weights. The column was eluted with 0.05 *M* Tris buffer, pH 7.4 containing 1 *mM* benzamidinium chloride and 1 *mM* iodoacetic acid at 18 ml/h and 6-ml fractions were collected. The positions of the marker proteins in the elution diagram of a plasma run are marked by arrows (—) UV absorbance of plasma run (O-O) elution of IgG-binder as estimated by single diffusion in agarose gel containing a pure IgG M component.

sure ultrafiltration in the cold, using an Amicon Diaflo cell with an UM10 membrane, dialyzed against 0.07 *M* phosphate buffer, pH 6.4, in the cold and then run on a 2.5 $\times$ 40 cm DEAE-cellulose column in the same buffer at 30 ml/h and at 4°C. 10-ml fractions were collected, tested for their light absorption at 280 nm and for their capacity to precipitate an isolated IgG M component in single and double radial diffusion. The first light-absorbing fractions precipitated the IgG M component and were pooled (fig. 2). This pool was then dialyzed at 4°C against 0.05 *M* Tris buffer, pH 7.4, containing 1 *mM* benzamidinium chloride and 1 *mM* iodoacetic acid and applied to a 2.2 $\times$ 10 cm column of polyclonal IgG bound to Se-

Table I. Purification of IgG Fc-binding protein from alkaline extract of type 15, group A streptococci

	Volume ml	Total protein weight mg amino acid residues	Total IgG- precipitating activity mg	Yield, %	Specific IgG- precipitating activity, mg IgG precipi- tated/mg protein	Purification (-fold)
Neutralized bacterial extract	27.5	150.4	4.1 ¹ 3.6 ²	100 ¹ 100 ²	0.027 ¹ 0.024 ²	1 ¹ 1 ²
Pool after DEAE- cellulose chromatography	7.1	20.2	3.0 ¹ 2.8 ²	73 ¹ 78 ²	0.15 ¹ 0.14 ²	5.6 ¹ 5.8 ²
Pool after immunosorption on IgG-Sepharose	3.1	0.63	0.43 ¹ 0.39 ²	10.5 ¹ 10.8 ²	0.68 ¹ 0.62 ²	25 ¹ 26 ²

¹ Measured by radial diffusion in agarose gel containing an IgG1(K) M component.

² Measured by radial diffusion in agarose gel containing an IgG4(K) M component.

Table II. Amino acid composition of the IgG Fe-binding substance

Amino acid	Concentration ¹ , mol/100 mol total amino acids
Aspartic acid	11.3
Threonine	6.4
Serine	7.1
Glutamic acid	18.5
Proline	< 0.2
Glycine	6.8
Alanine	15.5
Glucosamine	< 0.2
Galactosamine	< 0.2
Valine	2.1
Half-cystine	< 0.2
Methionine	< 0.2
Isoleucine	4.9
Leucine	13.0
Tyrosine	< 0.2
Phenylalanine	< 0.2
Lysine	11.1
Histidine	0.4
Arginine	2.9

¹ Mean of three analyses of three batches of the IgG-binding substance.

pharose in the same buffer. The column was developed at 4°C with 45 ml/h and the 10-ml eluate fractions were tested for pH, conductance, and capacity to precipitate isolated IgG M components in single radial diffusion. The column was eluted stepwise with the starting Tris buffer, with the same buffer containing 0.5 M NaCl, with 0.1 M sodium acetate buffer, pH 3.5, containing 0.5 M NaCl, and finally with 0.1 M glycine buffer, pH 2.2, containing 0.5 M NaCl. In all buffers 1 mM iodoacetic acid and 1 mM benzamidinium chloride were included. Fractions precipitating pure IgG M components were eluted only with the sodium acetate buffer. The acetate eluate was neutralized, dialyzed against 0.05 M Tris buffer, pH 7.4, containing 1 mM benzamidinium chloride and 1 mM iodoacetic acid and then concentrated to 3 ml. This solution was used for physicochemical and immunochemical studies of the IgG-binding substance.

Table I gives the percentage yields of the IgG-binding substance and the specific IgG-precipitating activity of the different solutions during a typical isolation procedure.

Physicochemical Properties. The IgG-binding sub-



Fig. 5. Agarose gel electrophoresis at pH 8.6 of (left to right) normal human plasma, isolated streptococcal IgG-binding protein, and unfractionated streptococcal extract.

stance was analyzed for its amino acid content with the result shown in table II. The substance contained considerable amounts of glutamic acid, alanine, leucine, aspartic acid and lysine while no proline, methionine, tyrosine, phenylalanine, cystine, glucosamine or galactosamine could be detected. The concentration of the substance in the solution expressed as amino acid content was found to be 0.20 mg/ml.

When the substance was investigated by SDS-polyacrylamide gel electrophoresis it formed a dense cluster of bands at a position corresponding to an apparent molecular weight of 29,500. Reduction of the substance did not change its appearance in the gel (fig. 3).

To estimate the hydrodynamic volume of the IgG-binding substance it was applied to a column of Ultrogel AcA44 which had been calibrated [20, 22] with human plasma proteins of known molecular weight. As can be seen from figure 4, only one IgG-p recipitating peak was eluted and at a position between those of IgA and IgG. The substance migrated in the γ_1 zone on agarose gel electrophoresis (fig. 5).

Immunochemical Properties. When the solution of the IgG-binding substance was analyzed for its capa-

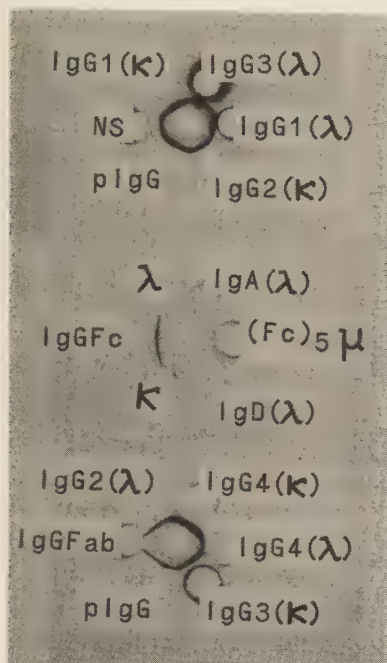


Fig. 6. Double radial diffusion of a solution of the isolated streptococcal IgG-binding protein and various pure immunoglobulins in dextran-containing agarose gel. 8 μ l of a solution of the IgG-binding protein with a peptide concentration of 0.20 mg/ml was applied in the central wells and 10 μ l of each immunoglobulin solution, at a concentration of 10 mg/ml, was applied in the peripheral wells. p IgG = Polyclonal human IgG; IgG Fab and IgG Fc = Fab and Fc fragments of polyclonal human IgG; NS = normal undiluted human serum. An (Fc)₅ μ fragment was used instead of native isolated IgM M components since all such available M components reacted with anti-IgG sera in double radial immunodiffusion.

city to precipitate various immunoglobulins in double radial diffusion the results shown in figure 6 were obtained. The substance precipitated all ten tested IgG M components of all subclasses and types as well as polyclonal IgG and normal human plasma forming a pattern of immunochemical identity. It also precipitated IgG Fc but not Fab fragments in such experiments. It did not precipitate IgA or IgD components; neither did it precipitate free γ or κ chains or (Fc)₅ μ fragments.

When the IgG-binding substance was diffused radially into agarose gel slabs containing pure IgG M

components, circular precipitation areas were produced (fig. 1). The size of the area was directly proportional to the amount of the IgG-binding substance applied. If the amount of IgG corresponding to the precipitation area was used to calculate the specific IgG-precipitating capacity of the IgG-binding substance it was found that 1 mg of the substance precipitated 0.6–0.7 mg IgG (table I).

No precipitates were produced when the solution of the isolated IgG-binding substance was tested in double radial immunodiffusion against three different antisera specific for streptococcal type M 15 protein. In contrast, the crude streptococcal extract produced one heavy precipitation line with each of the antisera.

The solution of the IgG-binding substance (0.2 mg/ml) was tested for its capacity to agglutinate sensitized human red blood cells. The Ri-coated cells were agglutinated in a titer of 1:200,000; the KM-coated cells in a titer of 1:800; and the HUN-coated cells in a titer of 1:2,400. Uncoated cells were not agglutinated by the solution. Todd-Hewitt broth, with or without the protease inhibitors used in this investigation, did not agglutinate the coated or the uncoated red blood cells.

Discussion

Binding of human IgG to streptococci by means of its Fc portion has been known for several years [17]. Although it has proved possible to extract [3, 25] and partly purify [5] IgG-binding material from streptococci, no reports concerning the isolation and physicochemical nature of the IgG-binding factor(s) have been published. Such an isolation of an IgG-binding factor was accomplished in this work by a combination of alkaline extraction of bacteria and ion-exchange and bioaffinity chromatography. The crucial step for the successful isolation proved to be the use of protease inhibitors: When protease inhibitors were not used it was found impossible to construct a reproducible isolation procedure, and the IgG-binding activity of various solutions often decayed rapidly. In contrast, when a mixture of several protease inhibitors of different specificities was added directly at the extraction of the bacteria and the entire purification process was carried out at 4°C in the presence of protease inhibitors these problems could be avoided. It remains to be investigated, however, whether each of

the four inhibitors was necessary in order to preserve IgG-binding activity.

It was not possible to calculate a yield for the entire solution process from bacteria to pure substance since no method is available for quantitation of the IgG-binding substance on cell walls. However, by quantitation of the IgG-binding substance in solution by its precipitation in agarose with isolated IgG M components it was found that the alkaline extract of 60 g bacteria (wet weight) contained approximately 5.9 mg of the IgG-binding substance and that about 11% of it was recovered in a pure state.

Although 100% of the isolated material bound to immobilized monoclonal or polyclonal IgG it did not display complete charge and molecular size homogeneity. However, the heterogeneity demonstrated by SDS-polyacrylamide and agarose gel electrophoresis was relatively restricted, especially in comparison with that of the crude starting material.

IgG-binding substances isolated from several alkaline bacterial extracts displayed identical physicochemical and immunochemical properties. It should be pointed out, however, that the yield of the substance varied slightly. On some occasions small amounts of IgG-binding substance were eluted from the immunosorbent column with eluants other than the acetate buffer, whereas the bulk was invariably eluted with this buffer. The reasons for these differences may conceivably be related to variability in the cultivation or extraction conditions.

The complete structure of the isolated IgG-binding substance can only be revealed by further investigations. It seems to be clear from our data that an integral part of its structure, necessary for its IgG-binding capacity, is one, or more, polypeptide chains. Thus, the substance loses its IgG-binding capacity when treated with proteolytic enzymes [4], releases appreciable amounts of amino acids upon acid hydrolysis and stains with common protein stains. The material does not seem to contain any peptidoglycan since it was devoid of glucosamine [28]. Similarly, as lipoteichoic acid would be retained by the ion-exchange resin used [16], its presence in the purified preparation is unlikely.

The IgG-binder displayed little, if any, light absorption at 280 nm, in congruence with the absence of aromatic amino acids shown by chemical analysis. Therefore, methods other than simple UV light absorption, have to be used to trace the material through a purification procedure. We have used single radial

diffusion in agarose gel slabs containing isolated IgG M components to quantitate the IgG binder in various fractions and double radial diffusion against pure IgG M components as a qualitative test.

The amino acid composition of the IgG binder differs from that of protein A from *S. aureus* [29] and from those of some investigated M, T and R proteins from group A streptococci [1, 15, 21] being distinguished especially by its high content of glutamic acid and relatively low content of aspartic acid, proline and phenylalanine.

Although the IgG binder exhibited an apparent molecular weight of about 30,000 as determined by SDS-polyacrylamide gel electrophoresis, gel chromatography in associative buffers revealed the hydrodynamic volume to be close to that of IgA. This means that the parent molecule is either remarkably elongated or forms oligomers in associative buffers. Oligomerization or elongated form of the molecule may be a prerequisite for its ability to precipitate IgG in gels.

In the present precipitation studies, agarose gels containing dextran were used. Dextran, like polyethylene glycol, has previously been described to induce precipitation of immune complexes soluble in buffers not containing such precipitation augmenters [11]. Dextran seemed to enhance precipitation also of the complexes formed between IgG molecules and the streptococcal IgG binder, as weaker precipitates occurred in the absence of dextran. The primary interaction between IgG molecules and the IgG binder does not, however, require the presence of dextran, as evidenced by the hemagglutination and bioaffinity experiments in which dextran was not used.

Whether the streptococcal material, like staphylococcal protein A [10, 12], reacts with some IgA, IgM and IgE molecules remains to be studied. In accordance with protein A [18], the streptococcal substance displayed a seemingly unrestricted reactivity for human IgG1, 2 and 4. However, the specificities for IgG3 of protein A and the streptococcal substance obviously differ, since the IgG3 proteins included in the present investigation did not react with protein A [13].

Further studies on the physicochemical structure and biological role of the IgG-binding substance are underway. Interestingly, the substance was recently demonstrated to inhibit complement-mediated lysis of IgG-sensitized sheep red blood cells and shown to be an important virulence factor of group A streptococci [2].

Previous studies [4] have demonstrated that the

IgG-binding substance in crude type 15 streptococcal extract is specific for IgG Fc. It is evident that also the purified streptococcal substance reacts with IgG determinants not belonging to the antigen combining sites since it did not react with Fab but clearly did so with Fc fragments and with a number of tested IgG components. In double diffusion in gel, the substance precipitated human IgG M components of all four subclasses and of both light chain types, polyclonal IgG, IgG Fc and normal human serum. It did not precipitate IgG Fab, IgA or IgD M components, free κ or λ chains, or (Fc) μ fragments. Erythrocytes coated with IgG antibodies of subclasses 1 and 3 were agglutinated by the isolated streptococcal substance at high dilutions.

As a tentative designation of this streptococcal binder of IgG Fc fragments we would like to suggest SB-GFc.

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CHANGES IN VIRULENCE, M PROTEIN AND IgG Fc RECEPTOR ACTIVITY IN A TYPE 12 GROUP A STREPTOCOCCAL STRAIN DURING MOUSE PASSAGES

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Burova, L. A., Christensen, P., Grubb, R., Jonsson, A., Samuelsson, G., Schalén, C. & Svensson, M.-L. Changes in virulence, M protein and IgG Fc receptor activity in a type 12 group A streptococcal strain during mouse passages. *Acta path. microbiol. scand. Sect. B*, 88: 199–205, 1980.

A type 12 group A strain (1800) was passaged serially through mice 25 times. The ability to survive in normal human blood dropped from a growth index of 52 after the first passage to 1 after four passages. After 14 passages the growth index increased again and stabilized above 30. The virulence for mice increased from a LD₁₀₀ of 10⁸ colony forming units (CFU) to 10–100 CFU after 7 passages and then remained constant. The M12 antigen disappeared after 4 passages as tested by immunodiffusion, electroimmunoassay and indirect bactericidal tests. Three antisera, raised in rabbits against strains originally belonging to types M3, M12 and M46 but devoid of type antigens after mouse passages showed high bactericidal indices against the 1800 strain after 14 or more passages on mice. Anti-type M1 serum was also found bactericidal for the passaged strains. The IgG Fc-receptor activity of the strain isolated after each mouse passage was tested in hemagglutination experiments with human red blood cells coated with »incomplete« anti-Rh and hot hydrochloric acid extracts of the strains. The capacity to agglutinate »Ripley«-coated cells increased gradually during the first 12 passages and subsequently the titres of the extracts stabilized between 1:160 and 1:320. The HUN coat, useful for detection of the G3m (5) marker gave titres increasing with the number of passages while the titres for IgG1 coats kept at 1:4 or below. On background of these results, the possible role of the IgG Fc-receptor as a virulence factor is discussed.

Key words: Streptococci; M protein; bactericidal test; human IgG Fc; anti-human Ig; Gm-markers.

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The laboratory methods recommended for the investigation of the virulence of group A streptococci include examination of mouse virulence and test of the ability of the strain to grow in normal human blood (13, 14, 17, 25). The hyaluronic acid capsule — and in particular — the type specific antigens or M proteins are virulence factors of recognized importance (12, 20). It is a widespread opinion that a strain which grows in normal human blood and is virulent for mice contains M protein,

also when extracts of the strain fail to precipitate with type-specific antisera. Nevertheless, only type-specific antibodies are generally assumed to promote phagocytosis in human blood. It is a matter of discussion whether group A streptococcal strains might be entirely devoid of M protein or substances closely resembling the M protein (9, 10). Hence, the term »absence of M protein« refers to the functional absence of M protein as defined by the tests for virulence referred to above

In addition to the M protein and the streptococcal capsule, some other, hitherto unknown structures might be important for streptococcal virulence (1). Recently, it was found (5) that many group A streptococci have receptors for the Fc fragment of IgG: the solubilized receptors display a variation in affinity between streptococcal types, e.g. with respect to reactivity for the human IgG1 and IgG3 subclasses (3). It remains to be investigated whether these receptors, which are distinct from the M protein (4), could interfere with antibody-mediated phagocytosis.

One of the usual procedures for reestablishing the M protein production of a strain is to pass the bacteria serially through mice and simply follow the presumed acquisition of M antigen by testing these passaged strains for growth in normal human blood (13). The present investigation concerns the changes observed in a type 12 group A streptococcal strain during serial mouse passages. In contrast to the concepts about M protein and virulence referred to above it was found that the type 12 antigen was lost during the passages although the virulence for mice and the ability to grow in human blood increased. During the passages, the bacterial content of receptors for the Fc-part of human IgG also increased.

MATERIALS AND METHODS

Bacterial Strains and Extracts

Group A streptococci type 12 (strain 1800, from the collection at the Institute of Hygiene and Epidemiology, Prague) were used throughout the study. Hot hydrochloric acid extracts were prepared from over night cultures of the streptococci on 300 ml Todd Hewitt broth as previously described (3).

Mouse Passages of the M12 Strain

This method has been described previously (2). In brief, 0.5 ml of over night culture of streptococci was injected intraperitoneally in white mice of 10–12 g. After each mouse had died from septicemia, a suspension of the spleen was injected intraperitoneally in a new mouse etc. After each mouse passage, the strain was isolated and designated according to the number of mouse passages performed before isolation, e.g. the strain isolated after 15 passages was called M12/15.

The experiments described in this paper were confirmed in two repeated series of mouse passages with strain 1800.

Test of Streptococcal Strains for Mouse Virulence

White adult mice were inoculated intraperitoneally with 0.5 ml of tenfold dilutions, $1:10^{-1}$ to $1:10^{-8}$, of an over night culture of the test-strain. Five mice were injected with each suspension and the animals were observed for 3 days after which the number of deaths

was registered. The number of colony-forming units (CFU) killing all mice was calculated (LD_{100}).

Rabbit Sera

Two rabbit antisera to type 12 group A streptococci, strain 1800 were prepared, one in Leningrad (Le) and the other in Lund (Lu). Furthermore, antiserum to type M1 strain 40/58 was prepared in Leningrad. These sera were used unabsorbed. Rabbit M-typing sera against type 1 and type 12 group A streptococci from the Institute of Hygiene and Epidemiology, Prague (Pr) were also used as was anti-type 12 M-typing serum, kindly supplied by Dr. *El Kholy*, Cairo (Ca).

Antisera to three strains, originally types 3, 12 and 46 but devoid of type-specificity after hundreds of mouse passages (2), were also raised in rabbits. These sera were designated anti-3P, anti-12P and anti-46P, respectively. The immunization schedule for the production of these and other antisera used followed the description given by *Rotta et al.* (21).

The T-typing sera were purchased from Chemapol, Prague.

Bactericidal Tests

These tests were performed essentially as described by Lancefield (13) and Šrámek (25). In brief, a measured quantity of streptococci (the inoculum) was mixed with freshly drawn human blood, alone or in the presence of added serum, and incubated for 3 hours in rotating tubes. The growth index (25) indicating the degree of inoculum propagation in normal human blood, was calculated from the formula

$$\frac{\text{no. of CFU obtained with normal serum}}{\text{no. of CFU in inoculum}}$$

The bactericidal index (BI) indicates the degree of inoculum-growth inhibition in the presence of anti-streptococcal serum as compared to controls with normal serum (24). This index was calculated from the formula

$$\frac{\text{no. of CFU obtained with normal serum}}{\text{no. of CFU obtained with antiserum}}$$

The scale used for expressing BI values was: BI below 25, 0; 51–100, +; 101–200, ++; 201–500, +++; and above 500, ++++ (24).

Demonstration of M Protein in Electroimmuno Assay

M protein in the extracts of the passaged strains was demonstrated by electroimmuno assay (15). Twenty ml 1% (w/vol) agarose (Miles Ltd, England) in 0.075 M barbital buffer, pH 8.6 containing 2 mM calcium lactate was mixed with 0.8 ml anti-type 12 serum Lu and poured over a 205×110 mm glass plate. Circular wells taking 5 μ l extract were cut 80 mm from the projected anode. After application of the extract a current of 6 V/cm was applied for 16 h. The plate was stained with Coomassie Blue.

Tests for Streptococcal IgG Fc-Receptors

These tests were performed essentially as described previously (3). In brief, Rh-positive red blood cells were coated with «incomplete» anti-Rh antibodies from various individuals. The capacity of different dilutions of the extracts to agglutinate the coated cells was tested in microtiter plates. The anti-Rh Ripley (Ri) was a gift from Dr. Marion Waller, Richmond U.S.A.; this anti-Rh is exceptional as it is complement fixing and not limited to one subclass. Anti-Rh KM and 317 are useful for detection of the human IgG1 genetic markers G1m(1) and G1m(4), respectively. The anti-Rh HUN detects the IgG3 marker G3m(5).

Double Immunodiffusion-in-gel

Gel precipitation test were performed as earlier described (23).

Quantitation of Protein

The protein content of the extracts was quantitated by a modified Folin method (16).

RESULTS

Changes in the Virulence of the Streptococcal Strain During Mouse Passages

The growth index (which indicates the ability of a streptococcal strain to survive in human blood in rotating tubes) dropped from 52 after the first passage to one after four passages. The strains M12/5 to M12/13 also showed very low growth indices (Fig. 1). After 14 passages, the growth index again increased, to 40, and subsequently remained constantly high between 31 and 48 during the final passages (see Table 1).

After each passage, the isolated strains were also tested for mouse virulence. The results obtained showed increasing virulence during the first four passages; the LD₁₀₀ dropped from 10⁸ to 10³ CFU. During the following three passages the virulence was further increased. After the 7th passage the virulence stabilized and remained constant showing LD₁₀₀ between 10 and 100 CFU.

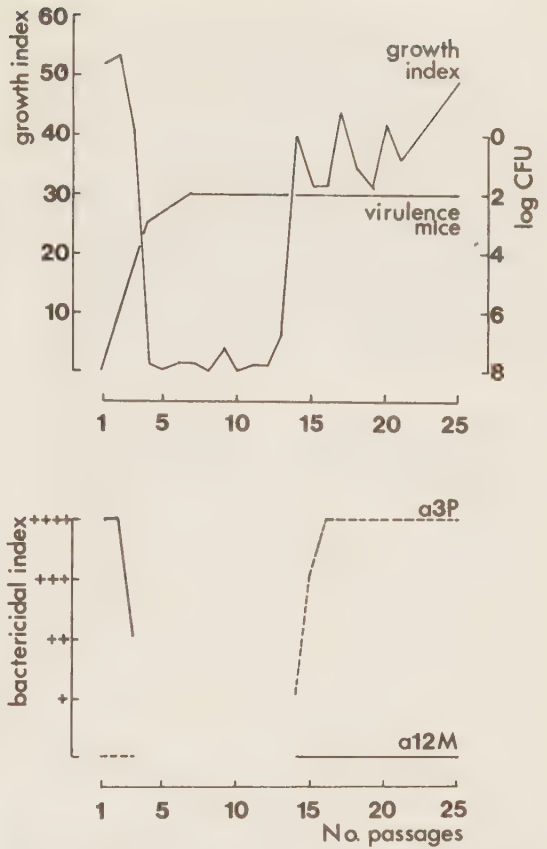


Fig. 1. Changes in virulence for mice and behaviour in bactericidal tests of a type 12 group A streptococcal strain during serial passages through mice. Abscissa: no. of passages performed. Ordinate: the virulence for mice given as the number of colony forming units (CFU) killing all of 5 mice injected with a suspension of the bacteria. The growth index, which indicates the capacity of the strain to survive in normal human blood was calculated according to Šrámek (25) The bactericidal index, which indicates the capacity of a serum to kill the streptococci in the presence of normal human blood was calculated according to Stollerman *et al.* (24); α3P indicates the results with a rabbit antiserum to strain 3P and α12M the results with a rabbit antiserum to type M 12 (the Le serum).

TABLE 1. Representative Bactericidal Tests with Some Selected Strains Obtained after Mouse Passages of Strain 1800

	Bactericidal test with strains					
	M12/1	M12/2	M12/3	M12/14	M12/19	M12/25
No. of CFU inoculated	105	93	93	55	86	106
Growth index	52	53	41	40	31	48
Bactericidal index						
-anti-type 12 serum	> 5488	1249	137	1	0	1
-anti-3P serum	0	1	1	85	> 2733	5168



Fig. 2. Electroimmunoassay with hot hydrochloric acid extracts of strain 1800 before and after passages through mice. Twenty ml agarose was mixed with 0.8 ml of the anti-type 12 serum Lu and poured over a glass plate. Extracts were placed in the circular wells and after application of the current for 16 hours and washing, the plate was stained with Coomassie Blue. »or« indicates the precipitate obtained with strain 1800, and 1, 2, 3 etc the results of testing strains M12/1, M12/2, M12/3 etc. The extracts of strains M12/10–M12/25 not shown on the picture did not give any precipitates.

Loss of Type-Specificity During the Mouse Passages

The Ca, Le and Lu anti-type 12 sera were compared by immunodiffusion in experiments with the hydrochloric acid extracts of the strains isolated after each of the 25 passages. All three sera gave a strong precipitate against each of the extracts of M12/1, M12/2, M12/3 as well as the original strain. When the precipitates were compared with one another, Ouchterlony type I reactions (interference with complete fusion) were obtained between all lines. This precipitate was not obtained with any of the extracts from the strains M12/4 to M12/25.

Figure 2 shows the results of electroimmunoassay quantitation of the M protein content in the hydrochloric acid extracts of the passaged strains. The height of the »rockets« increased during the first three passages. However, the M protein precipitate disappeared and did not recur after the fourth passage.

The protein content in the hydrochloric acid extracts of the passaged strains varied from 18 to 31 mg/ml. There was no correlation between the protein content of the extracts and the number of passages performed.

T-typing (11) of the passaged strains M12/1–4, 7, 9, 10 and 25 was performed and all these strains were found to belong to T-type 12. The other strains were spontaneously agglutinating and T-typing could therefore not be performed.

The loss of M12 protein was also confirmed in the indirect bactericidal test using the anti-type 12 sera Le and Pr. The bactericidal index (which indicates the ability of an immune serum to kill a streptococcal strain in the presence of fresh blood (24)) was estimated as ++++ after two passages and ++ after three. Because of the low growth index, it was not possible to determine the bactericidal effect of the anti-type 12 sera on strains M12/4 to M12/13. The strains M12/14 to M12/25 gave bactericidal indices with anti-type 12 serum estimated as one (Fig. 1).

Recently, a suitable donor for bactericidal tests with strain M12/6 (growth index 53) was found (Havlíček, personal communication). The strain was inhibited neither by anti-M12, nor by anti-M1 serum.

Test of the Passaged Strains for Streptococcal IgG Fc-Receptors

The extracts of the passaged strains were tested for capacity to agglutinate Ri-sensitized human red

TABLE 2. Agglutination of O Rh + Red cells Coated with Human IgG Anti-Rh by Lancefield Extracts of Type 12 Group A Streptococci Subjected to Mouse Passage

Human IgG coat	Genetic marker detected by the coat	Titre of the extract of type 12 group A streptococci after mouse passage no.						
		0	6	7	11	13	23	25
Ri: (1:15)*		1:10	1:80	1:20	1:160	1:160	1:320	1:320
KM: (1:5)*	G1m(1)	1:4	0	0	1:4	1:4	1:4	1:4
317 (1:5)*	G1m(4)	1:2	0	0	0	0	0	0
HUN (1:10)*	G3m(5)	1:10	1:10	1:10	1:20	1:20	1:40	1:80

* Indicates the dilution of the anti-Rh at coating.

TABLE 3. *Summary of the Results of Bactericidal Tests and Virulence Tests*

Strains	Virulence for mice	Growth index ^a	type 12M protein	Bactericidal index using	
				anti-M12 serum	anti-3P 12P and 46P sera
M12/1 -3	low/intermediate	high	present	high	one
M12/4 -13	high	zero	absent	n.d. ^b	n.d. ^b
M12/14-25	high	high	absent	one	high

^a the same blood donor used throughout the study.

^b not done because of low growth index.

cells. The titres did not exceed 1:10 in the extracts of the original strain, M12/1, M12/2 and M12/3. The titres of the extracts of M12/5 -9 varied between 1:20 and 1:80. From M12/11 to M12/25 the titres increased and stabilized between 1:160 and 1:320 (Table 2).

The hemagglutinating capacity of nine of the extracts was tested in experiments with red cells coated with KM, 317 or HUN »incomplete« anti-Rh. The HUN coat useful for detection of the G3m(5) marker, gave titres increasing with the number of passages, while the titres for the IgG1 coats, KM and 317, remained at 1:4 or below (Table 2).

Bactericidal Effect of Antisera against Strains Repeatedly Passaged in Mice and of Antiserum to Type M1 Group A Streptococci

Three antisera, anti-3P, anti-12P and anti-46P, were tested for bactericidal effect on the passaged strains of type 12 group A streptococci. The three antisera had been raised in rabbits by immunizing with strains originally belonging to types M3, M12 and M46 but devoid of type antigens after mouse passages. The effect of anti-3P in indirect bactericidal test is shown in Fig. 1 and in Table 1; similar results were obtained with anti-12P, anti-46P and anti-M1 sera. These sera had no effect in the test with M12/1-3. However, a high bactericidal index was found for the strains M12/14-25.

DISCUSSION

During the mouse passages, several characteristics changed in the type 12 group A streptococcal strain which confirm and extend previous findings (2). Table 3 summarizes the results of some of the experiments. It appears that three major phases, represented by strains M12/1-3, M12/4-13 and M12/14-25, occurred during the passages. Clear-

cut differences were observed between strains M12/1-3 as compared to strains M12/14-25, including disappearance of M12 antigen, concomitant increase in mouse virulence and susceptibility in bactericidal test using antisera against heterologous types.

The disappearance of M12 antigen after the fourth passage was observed in the precipitation studies and also in the indirect bactericidal test using anti-type M12 sera. The mouse virulence increased after four passages and remained high during the following passages. In contrast to the increasing mouse virulence, the ability of the strain to survive in normal human blood diminished after the fourth passage and did not recur until after 14 passages, although each strain was tested with blood from the same human donor. Discrepancies between virulence for mice and behaviour in bactericidal tests as found for strains M12/4-13 have also been noted in some group A streptococci by Todd (26) and Lancefield (13).

Conceivably, at least three explanations may be given for the changes in the characteristics of strain 1800 during mouse passages:

1) The strain 1800 might be a mixture of two M-types, e.g. M12 and M1, since anti-M1 serum was bactericidal for strains M12/14-25. Hence, the M1 strain might have been selected because of its higher mouse virulence. This would also be consistent with the finding that M1 has high IgG Fc-receptor activity (6). However, this hypothesis implies also that the M3 and M46 strains were contaminated with type M1 since antisera raised against these strains after mouse passages were bactericidal for strains M12/12-25. This objection is valid also for suspected »contamination« of the type 12 strain with any other M type. Studies involving clones from single cells of strain 1800 are in progress in order to further exclude the possibility of contamination.

2) The changes in strain 1800 could be caused by

a shift in M-type. *Maxted & Valkenburg* (18) described an epidemic in which type 12 group A streptococci showed »antigenic drift« towards type 22. However, »antigenic drift« occurring during the mouse passages would imply that the M antigens of three types, M3, M12 and M46 had changed to one and the same type antigen during the passages i.e. type 1, since anti-M1, 3P, 12P and 46P were all bactericidal for strains M12/14–25.

3) Mouse passages of some group A streptococci of defined M type might occasionally lead to selection of variants which lack M-antigen but have a hitherto unknown virulence factor in common. *Wittner* (27) using strains which had been serially passed through mice found that purified M proteins had the capacity to absorb opsonic antibody from a variety of heterologous antisera prepared against whole cells or purified M proteins. This absorption also separated passive mouse protecting antibodies and passive hemagglutinating antibodies from opsonic antibodies. The author interpreted these observations as caused by shared determinants among M proteins of different serotypes. However, *Fischetti* (7) (who did not use mouse passaged strains) found only limited structural relatedness among three purified M proteins examined and found no evidence of an anti-phagocytic activity caused by a peptide sequence common to M proteins. *Fleck* (8) found anti-type 6 and anti-type 1 serum to protect mice against infection with type 18 streptococci, suggesting »an antibody other than M, which could protect mice and enhance phagocytosis«.

Several reports have indicated the existence of other virulence factors than the hyaluronic acid capsule and M protein. *Becker et al.* (1) found that mouse passaged strains were rich in M protein and hyaluronic acid and concomitantly virulent for mice. However group A streptococci which had been selected after incubation with human blood were not virulent for mice although their content of M protein and hyaluronic acid was as high as found in mouse-passaged strains. On background of the discrepancy with respect to mouse virulence, *Becker et al.* (1) suggested the existence of as yet unidentified streptococcal virulence factors, in addition to M protein and capsule. We have found that the M12/25 strain retained the virulence for mice also after and during injection in the test animals of hyaluronidase, thereby removing the capsule of the streptococci (*Burova & Ryc*, unpublished observation).

Any pathogenic significance of the streptococcal receptors for human IgG Fc-fragment has not so far been shown. However, increase in reactivity for IgG Fc-fragment was concomitant with increase in mouse virulence in strains passed on mice. In this

context, it is of interest that *Perkins & Hahn* (19) found the phagocytosis of group A streptococci in human blood to be enhanced by antibody fragments derived from rabbit immunoglobulins by pepsin digestion as compared with undigested antibody. *Saito* (22) digested rabbit streptococcal anti-M antibody with papain; the digested univalent antibody retained the indirect bactericidal activity *in vitro* and was as effective as intact anti-M antibody. Thus, the Fc-fragment of IgG does not contribute to the phagocytosis of group A streptococci in contrast to the phagocytosis of other bacteria (19).

In conclusion, loss of original M antigen, concomitant with increasing virulence for mouse has been demonstrated in a type 12 group A streptococcal strain during mouse passages. Antisera against type M1 and three other heterologous types subjected to mouse passages were found bactericidal for the strains obtained from passages of the original type 12 strain. The nature of the structure responsible for virulence of the passaged strain is not known. However, the possible role of the streptococcal IgG Fc-receptor should be considered.

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IgG F(ab')₂ OF RABBIT ANTI-M SERA BUT NOT THE UNFRACTIONATED SERA ARE BACTERICIDAL FOR SOME GROUP A STREPTOCOCCI WITH IgG Fc-RECEPTOR ACTIVITY

Opsonic Effect Ascribable to Anti-IgG

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Schalén, C., Burova, L. A., Christensen, P., Grubb, R., Samuelsson, G. & Svensson, M.-L.: IgG F(ab')₂ of rabbit anti-M sera but not the unfractionated sera are bactericidal for some group A streptococci with IgG Fc-receptor activity. Opsonic effect ascribable to anti-IgG. Acta path. microbiol. scand. Sect. C, 89: 247-252, 1981.

We have earlier reported on a group A streptococcal strain, type M12, which upon serial mouse passage acquired IgG Fc-receptor activity but lost the M-antigen. The passaged strain, 12P, was highly virulent for mice and grew well in normal human blood. The present study particularly concerns the opsonic effect on 12P of rabbit anti-M3, anti-M12 and anti-12P sera, as well as the corresponding IgG F(ab')₂. Indirect bactericidal tests showed that the homologous anti-12P serum and IgG F(ab')₂ were opsonic. The anti-M3 and anti-M12 had no effect on 12P; surprisingly, however, IgG F(ab')₂ isolated from these sera displayed a clearcut opsonic activity. Data are presented which indicate that these »paradoxical« results can be explained by the binding of IgG F(ab')₂ with anti-IgG specificity to human IgG, linked to the streptococcal surface through Fc-receptors. Only anti-12P serum, or IgG F(ab')₂, were protective for mice on challenge with strain 12P.

Key words: streptococci; phagocytosis; anti-immunoglobulins; antistreptococcal serum; IgG; F(ab')₂; Fc-fragments.

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Opsonization is an important defence mechanism against group A streptococci (9). The opsonic activity of a serum is usually investigated by the classical bactericidal test; fresh human blood is mixed with the serum and living bacteria, and the number of streptococci surviving incubation is then recorded (10, 11, 13, 20). The mouse protection test measures the capacity of a serum to protect mice upon injection of living streptococci (9-11). The opsonic effect of a serum is generally held to be

exerted only through antibodies directed against the M antigens (9).

In a recent study, we followed the changes in virulence of a type 12 group A streptococcal strain during mouse passages (3). It was found that the virulence for mice increased, as did the capacity of the strain to grow in fresh normal human blood, although the M12 antigen was lost. The disappearance of the M12 antigen was demonstrated immunochemically and by the lack of opsonic effect of anti-type 12 serum in bactericidal tests. This

strain was designated 12P. During the passages, an increase was also found in the capacity of strain 12P to bind IgG via the Fc-part (3).

F(ab')₂ of IgG exert an opsonic effect for group A streptococci comparable to that of the native antibody (16, 18). The present paper concerns the opsonic activity of such fragments of rabbit immune sera raised against the mouse-passaged strain 12P and two M-antigen positive, non-passaged strains, M3 and M12. Apparently paradoxically, it was found that F(ab')₂ derived from the anti-M sera were opsonic for 12P in spite of the fact that the unfractionated sera were not.

MATERIALS AND METHODS

Streptococcal Strains and Extracts

The Group A strains, type M3 (No. 4/44) and M12 (No. 1800) were obtained from the collection at the Institute of Hygiene and Epidemiology, Prague. The strain 12P was derived from the corresponding M type by serial passage through mice, and thereby deprived of the M12 antigen (3). Hot alkaline extracts of the strains were prepared from overnight cultures in Todd-Hewitt broth (5).

Rabbit Immune Sera

The rabbit antisera raised against strains M3, M12 and 12P have been described earlier (2, 3). The sera were used unabsorbed unless otherwise stated. Rabbit antisera to human IgG and mouse immunoglobulins, and porcine antiserum to rabbit IgG, were purchased from DAKO, Copenhagen, Denmark.

Human, Rabbit and Mouse IgG

Commercial human IgG was purchased from AB Kabi, Stockholm, Sweden. Normal rabbit and mouse IgG were prepared from the sera of non-immunized animals by ammonium sulphate precipitation and filtration on a Sephadex G200 column (Pharmacia, Uppsala, Sweden). The fractions containing IgG as tested by the above-mentioned antisera were pooled.

Rabbit IgG F(ab')₂ -Fragments

IgG from the antistreptococcal sera was precipitated with 40% saturated ammonium sulphate. After dialysis against 0.1 M sodium acetate, pH 4.5, and determination of the protein concentration (12), pepsin (Worthington, New Jersey, USA) 3000 U/mg was added to the IgG at an enzyme/substrate ratio of 1:20 and the sample incubated at 37 °C for 18 hours. The digestion was stopped by dialysis against phosphate-buffered saline (PBS, 0.03 M phosphate, 0.12 M NaCl, pH 7.2). The sample was then passed on a column of AcA 22 (Pharmacia). The fractions containing IgG were pooled and concentrated.

Isolation of IgG F(ab')₂ Directed against Human IgG

Commercial human IgG, 100 mg, was coupled to 10 g of CNBr-activated Sepharose 4B (Pharmacia) by the

procedure recommended by the manufacturer. Two ml of IgG F(ab')₂, 5 mg/ml, from an anti-M3 serum was applied on the immunosorbent column. The column was then washed with PBS until the eluate no longer showed light absorption at 280 nm. The protein-containing fractions from the washings were pooled and concentrated to 1.5 ml, containing 3 mg/ml of protein (pool A). The column was then eluted with 0.1 M glycine, pH 2.2. The eluate was concentrated to 1.5 ml and dialyzed against PBS; the protein concentration was 0.4 mg/ml (pool B).

Agglutination and Agglutination Inhibition Tests

The streptococcal extracts were investigated for IgG Fc receptors in hemagglutination tests using human rhesus-positive O red blood cells coated with the Ri or KM anti-Rh antibodies as described (4). The sensitized cells were also used to detect IgG F(ab')₂ with anti-IgG specificity as were SSRBC (1 per cent (vol/vol) sheep red cells sensitized with the IgG fraction of rabbit anti-sheep red cell antibodies) (1). Various IgG preparations were tested for inhibitory capacity in hemagglutination inhibition tests with KM-sensitized cells and IgG F(ab')₂.

Absorption of IgG Preparations with SSRBC

IgG F(ab')₂, 1.2 mg/ml, of anti-M12 serum was absorbed with 1:10 volume of packed SSRBC at 37 °C for 30 min; the supernatant after centrifugation for 30 min at 3000 g was recovered.

Double Radial Diffusion

Double radial diffusion of IgG F(ab')₂ against aggregated human IgG was performed as described previously (19).

Test for IgG Fc-Receptors on Intact Streptococcal Cells

The presence of surface IgG Fc-receptors on the streptococci was tested by determining the percentage of 2.5 µg ¹²⁵I-labelled IgG1-myeloma protein bound to a standard suspension of cells (6). In inhibition experiments, various unlabelled IgG preparations were added to this test system.

Bactericidal Tests

These tests were performed as described previously (3). In brief, an inoculum of 40 µl, containing 50–100 colony-forming units (CFU) of the strain to be tested, 20 µl of the antiserum diluted 1:3 (or 24 µg of IgG F(ab')₂) and 300 µl of freshly drawn heparinized human blood were mixed and the tubes incubated in a roller for 3 h at 37 °C, followed by recording of the number of CFU. Two human donors were used.

The growth index (21) expressed the ability of a strain to grow in normal human blood. The bactericidal index of an antiserum was calculated by dividing the number of CFU after incubation of the strain in normal human blood, by the number of CFU after incubation with the antiserum in the presence of normal human blood (20).

Mouse Protection Tests

White adult mice (10 g body weight) were injected subcutaneously with 1 ml of IgG F(ab')₂, 300 µg/ml, or

1 ml 1:10 dilutions of the immune sera. One hour later, different dilutions of an overnight culture of strain 12P were injected intraperitoneally. Two mice were injected with 0.5 ml of each tenfold dilution from 10^{-2} to 10^{-7} .

RESULTS

Opsonic Effect of Native Streptococcal Sera and their IgG F(ab')₂ in Bactericidal Tests

The sera anti-M3, -M12 and -12P were investigated in indirect bactericidal tests for capacity to opsonize the three strains, M3, M12 and 12P (Table 1). Each of the strains was only opsonized by the homologous serum, while the heterologous sera were without any effect.

The opsonic effect of IgG F(ab')₂ prepared from the three sera is also shown in Table 1. The M-

strains were exclusively opsonized by IgG F(ab')₂ taken from the homologous sera. In contrast, IgG F(ab')₂ from all three antisera showed opsonic effect for the 12P strain; the bactericidal index of IgG F(ab')₂ from anti-12P, however, was 10 times higher than that of IgG F(ab')₂, isolated from the anti-M sera. Thus, both anti-M sera were converted, from being devoid of effect on strain 12P, when native sera were used (BI < 1) to exert opsonic effect when the Fc-fragments were removed (BI 55 and 70, respectively).

Mouse Protection Tests with Native Antistreptococcal Sera and IgG F(ab')₂ thereof

The antisera and IgG F(ab')₂ isolated from the sera were also investigated for capacity to protect mice from lethal infection after challenge with living 12P streptococci. This strain was highly virulent for mice, showing a LD₁₀₀ of only 5 CFU. Neither the immune sera anti-M3 and -M12, nor IgG F(ab')₂ of these anti-M sera showed any protective effect (Table 2). The anti-12P serum exhibited as expected a strong protection, being effective for a challenge of 10,000 lethal doses; IgG F(ab')₂ prepared from this serum was protective for 1,000 lethal doses. Thus, IgG F(ab')₂ of the anti-M sera showed no opsonic effect in mouse-protection test, while they were clearly opsonic in bactericidal tests.

Test of IgG F(ab')₂ from Antistreptococcal Sera for Anti-Immunoglobulin Activity

IgG F(ab')₂ from the three antistreptococcal sera was tested for capacity to agglutinate various anti-Rh coated red blood cells. Cells coated with the Ri anti-Rh were agglutinated at concentrations of 0.18,

TABLE 1. *Effect in Indirect Bactericidal Tests of Rabbit Anti-Streptococcal Sera and IgG F(ab')₂ on Non-Passaged and Mouse-Passaged Streptococci*

Rabbit antistreptococcal serum (S) or IgG F(ab') ₂ -fragment (F)		Bactericidal index with strain		
		M3	M12	12P
Anti-M3:	S	1165	1	<1
	F	582	1	70
Anti-M12:	S	1	>4176	<1
	F	1	>4176	66
Anti-12P:	S	1	1	>1986
	F	<1	<1	662
Growth index		50	45	33

TABLE 2. *Mouse Protection Tests with Different Immune Sera and the Corresponding IgG F(ab')₂ Using the Mouse-Passaged Strain 12P*

Rabbit antistreptococcal serum (S) ¹ or IgG F(ab') ₂ -fragment (F) ³		Number of mice killed after receiving (CFU) ²						Total number of mice killed/mice injected
		5×10^5	5×10^4	5×10^3	5×10^2	5×10	5	
Anti-M3:	S	2	2	2	2	2	2	12/12
	F	n.d. ⁴	2	2	2	2	2	10/10
Anti-M12:	S	2	2	2	2	2	2	12/12
	F	n.d.	2	2	2	2	2	10/10
Anti-12P:	S	1	0	0	0	0	0	1/12
	F	n.d.	0	0	0	0	0	1/10

¹ Each mouse was given 1 ml serum diluted 1:10

² Two mice were given each of the streptococcal preparations

³ Each mouse was given 1 ml IgG F(ab')₂, 0.3 mg/ml

⁴ not done

TABLE 3. *Binding of Radiolabelled IgG Myeloma Protein by Strain 12P; Effect of Addition of Unlabelled Human, Rabbit and Mouse IgG*

Unlabelled IgG preparation added	Radiolabelled human IgG1 bound (in per cent) after mixture with unlabelled IgG		
	1 µg	10 µg	100 µg
Human IgG	35	26	4
Rabbit IgG	31	19	4
Mouse IgG	38	35	27

0.06 and 0.08 mg/ml of IgG F(ab')₂ from the anti-M3, -M12 and -12P sera, respectively. The KM-cells were agglutinated at concentrations of 0.82, 0.06 and 0.08 mg/ml, respectively. IgG F(ab')₂ from a normal rabbit serum agglutinated both Ri- and KM-coated cells at a concentration of 0.40 mg/ml. SSRBC were not agglutinated by any of the IgG F(ab')₂ at concentrations up to 4 mg/ml. IgG F(ab')₂ from anti-M12 and anti-12P but not from anti-M3 and normal rabbit serum precipitated human aggregated IgG in double radial diffusion.

The capacity of normal human, rabbit and mouse IgG to inhibit the agglutination of KM-coated cells by IgG F(ab')₂ from anti-M12 and anti-12P was also investigated. Human IgG was inhibitory at a concentration of 0.13 mg/ml, whereas both rabbit IgG (4 mg/ml) and mouse IgG (2 mg/ml) were without effect.

Test of Streptococcal Strains for IgG Fc-Receptors

Extracts of strains M3, M12 and 12P were tested for content of IgG Fc-receptors by the ability to agglutinate human red blood cells coated with the anti-Rh Ri. Neither extract of these M-strains was capable to agglutinate, while the 12P-extract was active at dilutions up to 1:160.

The 12P strain bound 40 per cent of the 2.5 µg radiolabelled IgG1 myeloma protein added to a standard amount of the bacteria, while M3 and M12 were without binding capacity. Various amounts of unlabelled IgG from man, rabbit or mouse were tested in competition experiments with radiolabelled human IgG. Both rabbit and human IgG inhibited the binding of radiolabelled human IgG to strain 12P, while mouse IgG was only slightly inhibitory (Table 3).

Absorption of IgG F(ab')₂ with Anti-IgG Activity: Effect on the Opsonization of Strain 12P

IgG F(ab')₂ of anti-M12 serum was absorbed with SSRBC, while (as mentioned) agglutinating Ri-

sensitized cells at a concentration of 0.06 mg/ml before absorption, the absorbed IgG F(ab')₂ did not agglutinate at a concentration of 1 mg/ml. In this experiment, the bactericidal index for strain 12P fell from 32 before absorption to 1 after absorption. The BI for strain M12 was 372 before and 237 after the absorption. In contrast, absorption of the IgG F(ab')₂ isolated from the anti-12P serum did not affect the opsonic capacity for strain 12P.

IgG F(ab')₂ of anti-M3 serum was filtered on a column of human IgG linked to Sepharose 4B. The fraction not binding to the column (pool A) agglutinated Ri-sensitized cells at 0.25 mg/ml, while the fraction eluted at low pH (pool B) agglutinated at 0.006 mg/ml. In this experiment, the BI before filtration was 37 for strain 12P, as compared with 1.5 and 20, respectively, for fractions A and B. In contrast, the BI of these fractions for M3 was 303 and 54, respectively.

DISCUSSION

IgG F(ab')₂ was found to retain the *in vitro* opsonic activity and the mouse-protective capacity of native antistreptococcal sera, thus confirming the results of Perkins & Hahn (16). The observation that the Fc-part is not needed for opsonization of group A streptococci was earlier received with scepticism (8), since it was thought that the Fc-part was necessary for complement activation. However, it has been shown that complexes of IgG F(ab')₂ and the corresponding antigen can activate complement via the alternative pathway (17).

The seemingly paradoxical finding in indirect bactericidal tests that removal of the Fc fragment converted IgG isolated from the anti-M sera to promote phagocytosis of strain 12P, calls for explanation. Our interest was directed to the question whether anti-immunoglobulins might be responsible for this bactericidal effect. Eyquem *et al.* (7) demonstrated as early as 1959 that many rabbit antistreptococcal sera contain anti-immunoglobulins; the anti-IgG may bind IgG from several animal species, including rabbit, and human IgG (15). Using erythrocytes coated with human IgG, we detected anti-IgG activity in all our IgG F(ab')₂ preparations by their agglutinating capacity. In contrast, none of the preparations agglutinated cells sensitized with rabbit IgG. Furthermore, human but not rabbit normal IgG could inhibit the agglutination of KM-coated cells by rabbit immune F(ab')₂. On the other hand, it was possible to absorb the anti-IgG from the preparations using cells sensitized with rabbit IgG. We conclude from these experiments that the anti-IgG found in our antisera

exhibited a higher affinity for human than for rabbit IgG.

It was found that absorption of IgG F(ab')₂ from anti-M12 serum with SSRBC abolished the capacity to opsonize the 12P strain, and to agglutinate cells coated by human IgG. Furthermore, the opsonic capacity for 12P of the IgG F(ab')₂ from anti-M3 serum, as well as the anti-IgG activity, was retained on a Sepharose 4B-human IgG column. The opsonic capacity and the anti-IgG activity were both eluted from the column at low pH, splitting antigen-antibody complexes. These results are not unique for 12P, since similar results have been obtained with a strain obtained after several mouse-passages of type M3 streptococci (unpublished observation). Our interpretation is that IgG F(ab')₂ from anti-M3 and anti-M12 sera, containing anti-IgG activity, was the prerequisite for *in vitro* opsonization of 12P.

Experiments were performed to elucidate the way in which anti-IgG might promote phagocytosis. The finding that the mouse-passaged, Fc-receptor-positive 12P – but not heterologous, non-passaged M-protein-positive bacteria – were opsonized by IgG F(ab')₂ from anti-M3 and anti-M12, indicated that the anti-IgG was active for streptococci expressing IgG Fc-receptors. Conceivably, the IgG F(ab')₂ with anti-IgG specificity might bind to human IgG, provided by the normal human blood used in the opsonization test and bound to streptococcal IgG Fc-receptors (Fig. 1). Such a

mechanism would also explain why these IgG F(ab')₂ were without effect in mouse protection tests, since mouse IgG binds only moderately to streptococcal IgG Fc-receptors (Table 3 and ref. 14). In contrast, rabbit IgG and human IgG were effectively bound to the receptors, as illustrated in the competition experiments with radiolabelled human IgG (Table 3). IgG F(ab')₂ is not bound to streptococcal Fc-receptors. Thus, removal of the Fc-part of rabbit IgG would result in binding of more human IgG to the streptococci during the bactericidal test, and thereby binding of rabbit IgG F(ab')₂ with predominant specificity for human IgG to the streptococcal surface.

The »conventional« opsonization of group A streptococci through anti-M antibodies (Fig. 1) was illustrated by the experiments with the strains M3 and M12 and the homologous antisera or IgG F(ab')₂. As proposed above, the opsonic effect on 12P exerted by IgG F(ab')₂ of the anti-M sera was ascribable to anti-IgG activity. The markedly lower bactericidal indices found in the latter case further underlined that different mechanisms were involved. Whether opsonization of 12P by the homologous antibodies, or IgG F(ab')₂, occurs due to an antibody specific for the Fc-receptor or through other mechanisms is currently investigated; the opsonic effect of the anti-12P IgG F(ab')₂ was apparently not dependent on anti-immunoglobulins, as absorption with SSRBC was without effect. As earlier reported, 12P is also opsonized by antisera to other mouse-passaged strains, and extracts of the strain are precipitated in double-diffusion in gel by absorbed anti-M1 serum (3).

In conclusion, anti-IgG of the IgG class present in rabbit anti-streptococcal sera may exert opsonic effect for certain mouse-passaged group A streptococci, expressing IgG Fc-receptors. The results of classical *in vitro* tests demonstrate that other antibodies than those directed against M-antigens may be indirectly opsonic for these strains.

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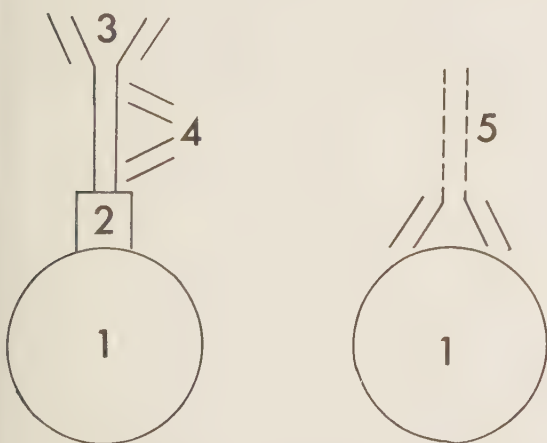


Fig. 1. Scheme of the proposed theory for action of anti-immunoglobulins in bactericidal tests and the action of »conventional« anti-M antibody.

1. The streptococcal cell; 2. The streptococcal IgG Fc-receptor; 3. Human IgG supplied by the donor blood used in the test; 4. IgG F(ab')₂ with anti-IgG specificity, prepared from anti-M serum; and 5. Anti-M antibody, or IgG F(ab')₂, »conventionally« attached through the antigen-binding sites to the streptococcal cell.

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